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The overall goal of this project has been to develop gene therapy strategies for breast cancer by translation of studies derived from the DF3/MUC1 gene. Our studies have demonstrated that defective recombinant adenoviral vectors containing the DF3/MUC1 promoter (bps -725 to +31) can be used to selectively express the herpes simplex virus thymidine kinase (HSV-tk) gene (Ad.DF3-tk) in DF3/MUC1 positive breast cancer cells in vitro and in animal model studies. On the basis of these findings, the replication defective adenoviral vectors containing the DF3/MUC1 promoter sequences have been used to selectively transduce contaminating breast cancer cells in human bone marrow and peripheral blood. Other studies have demonstrated that transduction of dendritic cells with replication defective adenoviral vectors expressing the DF3/MUC1 gene is effective for in vivo immunization against MUC1-positive tumor cells. These findings have provided the experimental basis for clinical trials of a novel vaccine against breast cancer. Finally, more recent work has involved the design of an adenoviral vector that is competent for replication in DF3/MUC1-positive, but not DF3/MUC1-negative, cells for the selective delivery of suicide genes to breast cancer cells in vivo.

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Gene Therapy of Breast Cancer: Studies of Selective Promoter/Enhancer-Modified Vectors to Deliver Suicide Genes

Contents

INTRODUCTION

Most patients with metastatic breast cancer succumb to their disease. Chemotherapy is often standard treatment for these patients; however, effectiveness of the available chemotherapeutic drugs is limited by lack of selectivity and a narrow therapeutic window. Gene therapy is a potentially novel approach to cancer treatment. In this context, transfer of suitable genetic material into a specific cell type (either tumor or host) can be used to alter the phenotype of the target cell. One such strategy is based on direct transfer of a "suicide gene" which encodes an enzyme, such as herpes simplex thymidine kinase (HSV-tk), that can activate a prodrug within tumor cells and thereby render the tumor cells sensitive to agents which are otherwise nontoxic to the cell. Another approach is to develop gene therapy strategies that induce active specific immunity against tumor-associated antigens. In the context of the delivery of a suicide gene, clinical efficacy will require a gene delivery system with high gene transduction efficiency and target cell specificity. By contrast, in the development of gene therapy based vaccines, transduction efficiency is less of an issue and target cell specificity is induced through the immune system.

Human adenoviruses are non-enveloped double-stranded DNA viruses with a genomic size of approximately 36 kb. The E1 gene deleted adenoviruses are replication defective and can be grown in a packaging cell line transformed with the E1a and E1b genes. Adenoviral vectors deleted at the E1 and E3 regions are capable of accomodating DNA inserts up to 8 kb. Moreover, adenovirus-

mediated gene transfer is a highly efficient means for delivery of genetic material into a wide spectrum of cells both in vitro and in animals. Although recombinant adenoviruses hold promise for in vivo gene therapy and are being tested clinically, one of the limitations of this vector system for cancer therapy may be the non-specific transduction of therapeutic genes into non-target cells. One strategy to circumvent this limitation would be to use a tumor-tissue specific/selective promoter or enhancer to direct the expression of a therapeutic gene in the desired target cells.

DF3 antigen (also designated MUC1 and episialin) is a member of a family of high molecular weight glycoproteins which are aberrantly overexpressed in most human breast cancers. We have previously shown that monoclonal antibody MAb DF3, prepared against a membrane enriched extract of a human breast carcinoma metastatic to liver, reacts with over 75% of primary human breast carcinomas. Other studies have shown that overexpression of the DF3 gene in human MCF-7 and ZR-75 breast cancer cells is regulated at the transcriptional level. Cloning and characterization of the 5' flanking region of DF3 gene has demonstrated that DF3 gene expression is mainly regulated by sequences between positions -598 and -485 base pairs upstream to the transcription start site. The DF3 protein is one member of the MUC1 family of carcinoma-associated antigens that contain variable numbers of highly conserved (G+C)-rich 60 base pair tandem repeats. A C-terminal region includes a transmembrane domain that anchors the antigen at the cell surface. Cell-cell interactions are reduced in cells transfected with the MUC1 cDNA. Other work has demonstrated that DF3 inhibits the recognition of targets by immune effector cells.

These findings have suggested that the DF3/MUC1 tumor-associated antigen may represent an attractive target for a vaccine against breast cancer.

BODY

Experimental Methods

1. Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/MUC1 promoter.

Cell culture. The MCF-7, ZR-75-1, BT-20, and MDA-MB231 breast cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The Hs578Bst myoepithelial cell line derived from normal breast tissue adjacent to a infiltrating ductal carcinoma (1) and the human T98G glioblastoma cell line were also obtained from ATCC. Cells were grown as monolayers in recommended culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were maintained in a 5% CO_2 humidified atmosphere.

Recombinant adenoviruses. Recombinant adenoviruses Ad.DF3-βgal and Ad.CMV-βgal derived from type 5 adenovirus (Ad 5), were produced by homologous recombination in the human embryonic kidney cell line 293 (2). The DF3 5' flanking region (-725 to -31) was inserted into Xho I and Spe I digested plasmid pCMVβgal (provided by Dr. R. Crystal, Cornell Medical Center, NY). The resulting plasmid pDF3βgal contains the E.coli β-galactosidase (lacZ) gene with the SV40 polyadenylation signal under the control of DF3 promoter and SV40 splice donor/acceptor signal, flanked by Ad 5 map units 0.0-1.3 and 9.3-17.3. To construct AD.DF3-tk, a 2.0 kb cDNA of HSV-tk was used to replace the lacZ gene in the shuttle plasmid pDF3βgal. The shuttle plasmids were cotransfected

by calcium phosphate precipitation into 293 cells together with pJM17 plasmid containing the adenoviral genome (kindly provided by Dr. Graham, McMaster University, Ontario, Canada). Recombinant adenovirus was isolated from a single plaque and expanded in 293 cells. The viral DNA was purified and analyzed by restriction enzyme digestion and by polymerase chain reaction (PCR). A pair of primers, 5'-TCTAGACTAGTGGACCCTAGGGTTCATCGGAG-3'and 5'-AACTCGAGGATTCAGGCAGGCGCTGGCT-3' was used to amplify and verify the presence of the DF3 promoter (-725 to -31) in the viral genome. Ad.CMV-βgal and AD.CMV-tk are structurally similar replication-deficient recombinant adenovirus in which the lacZ and HSV-tk genes, respectively, are under the control of cytomegalovirus (CMV) immediate-early promoter and enhancer. Large scale production of recombinant adenovirus was accomplished by growth in 293 cells and purification by double cesium gradient ultracentrifugation as described (3). The titers of purified adenovirus were determined by a spectrophotometer and by plaque assays.

Adenovirus infection in vitro. Twenty-four h after plating, cells were infected with adenovirus at a multiplicity of infection (MOI) of 10-50. 48 h later or at a specified time post infection, cells were evaluated for the expression of the reporter gene or evaluated for sensitivity to GCV.

Assays for β-galactosidase. Histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). Sections of fresh frozen tissue (12 μm) or cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) containing 1 mM

$MgCl_2$ for 10 min, rinsed with PBS, and then incubated with X-Gal (1 mg/ml), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS for 4 h.

Fluorescence-activated cell sorting (FACS) analysis. (i) Indirect immunofluorescent analysis of DF3 antigen. Cultured cells (1-2 x 10^6) were washed extensively with 1% bovine serum albumin (BSA) in PBS and incubated with MAb DF3 (1 μg/ml) or isotype identical control antibody mouse IgG (F-8765, Sigma Chemical Co., St. Louis, MO) at 4°C for 1 h, and then washed with 1% BSA/PBS. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F2012, Sigma) at 4°C for 1 h, washed and analyzed on a Becton Dickinson FACScan. Intensity of fluorescence was determined for 10,000 cells and compared with the fluorescence obtained using a non-reactive immunoglobulin of the same isotype. (ii) FACS-GAL assay (18). Briefly, 1 x 10^6 cells were suspended in 50 μl of serum-free culture medium and warmed to 37°C. An equal volume of 2 mM fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was added to each aliquot of cells. The cells and FDG were mixed rapidly and incubated for 1 min at 37°C. Thereafter, cells were washed once with 4 ml ice-cold PBS and maintained in ice-cold PBS until analysis on a Becton Dickinson FACScan.

Assays for GCV sensitivity in vitro. Adenovirus (Ad)-infected and noninfected cells were plated at 4 x 10^4 cells/well in 6 well plates. GCV was added to the culture medium at various final concentrations (0-250 μM). After 6-8 days of incubation, cells were washed with PBS and trypsinized. The number of viable cells were determined by trypan blue exclusion. Cell number was also assessed using a colorimetric cell proliferation (XTT) assay

that measures the mitochondrial dehydrogenase activity of viable cells . Results are expressed as a growth ratio of the nubmer of cells in plates containing drugs as a percentage of that in the corresponding drug-free controls.

In vivo gene transfer to human breast cancer xenografts.

Female athymic nude mice (Swiss-nu/nu, Taconic, Germantown, NY), 20-25 g, were used. For mice bearing MCF-7 or ZR-75-1 tumors, a single pellet of 17,β-estradiol (1.7 mg/60-day release, Innovative Research, Toledo, OH) was implanted subcutaneously one day before tumor inoculation. i) Subcutaneous tumor model. Cells (MCF-7, ZR-75-1 and MDA-MB231) in exponential growth phase (1×10^7 in 0.2 ml) were injected subcutaneously in the flanks of the animals. At 4 to 6 weeks after tumor implantation, up to 5×10^8 plaque-forming units (pfu) of purified recombinant adenovirus in 20 µl were injected into MCF-7, ZR-75-1, and MDA-MB231 xenografts, and into limb skeletal muscle. A Hamilton syringe with a 26 gauge needle was used for injection. The needle was coated with fine charcoal particles to mark the needle tract in order to verify co-localization of the expression of the reporter gene with the viral injection. Three days after adenoviral infection, the animals were sacrificed and expression of the reporter gene in tumor xenografts and host tissues was evaluated. ii) Intraperitoneal tumor model. MCF-7 cells (5×10^6) were injected i.p. for the development of intraperitoneal tumors (day 0). On day 4 and 5 after tumor cell injection, 1×10^9 pfu adenovirus in 0.5 ml PBS were injected i.p. into the mice. On day 7, the animals were treated with either saline or GCV (125 mg/kg) daily for 4 days by i.p. injection. The animals were sacrificed at 5-6 weeks after

tumor inoculation. Tumors were collected and weighed for each animal.

2. Selective transgene expression for detection and elimination of contaminating carcinoma cells in hematopoietic stem cell sources.

Cell lines. The MCF-7, ZR-75-1, BT-20 and SKBR3 breast carcinoma, the A549 lung carcinoma, DU145 prostate carcinoma, SKOV3 ovarian carcinoma and T98G human glioblastoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayers in recommended culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Human hematopoietic cells. Human PB mononuclear cells were isolated by Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation (d=1.077, 400 x g) from leukocyte-enriched leukopaks of healthy donors. Cells were suspended in RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Bone marrow was obtained from filters used to prepare harvested marrow from normal donors and the mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. Bone marrow stromal cells were isolated by adherence (4).

$CD34^+$ cells were isolated using the Ceprate LC cell separation system (CellPro Inc., Bothell, WA). In brief, BM cells were incubated with a biotinylated mouse anti-CD34+ MAb, washed and then passed through an avidin column. Nonadsorbed cells were

removed by washing, and adsorbed cells were eluted from the column. The enriched cells (80-90% CD34+) were maintained in Iscove's MEM containing 12.5% FBS, 12.5% horse serum and 1 μM hydrocortisone.

Antibody reaction and fluorescence-activated cell sorting (FACS) analysis. Monoclonal antibodies (MAbs) used were specifically reactive with the cell surface antigens: CD3 (T3, Coulter, Miami, FL), CD13 (L138, Becton Dickinson), CD19 (B4, Coulter), CD34 (Becton Dickinson, San Jose, CA), CD51 (integrin αv, clone 1980, Chemicon Inc., Temecula, CA), integrin αvβ3 (LM609, kindly provided by Dr. David Cheresh, Scripps Research Institute, CA) and integrin αvβ5 (5) (Clone B5-IA9, generously provided by Dr. Martin E. Hemler, Dana-Farber Cancer Institute). Cells were incubated with antibody for 30 min on ice. If the antibody was not directly conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), a secondary antibody conjugated with FITC or PE (SIGMA, St. Louis, MO) was used for indirect fluorescence labeling. Cells were then washed and evaluated by flow cytometric analysis.

Recombinant adenoviruses. Ad.CMV-βgal, Ad.CMV-tk (6), and Ad.CMV-Luc (kindly provided by Dr. Robert Gerard, University of Texas) (7) are replication-deficient recombinant adenoviruses in which the luciferase, β-galactosidase, and HSV-tk genes, respectively, are under control of the cytomegalovirus (CMV) immediate-early promoter and enhancer. Ad.DF3-βgal and Ad.DF3-tk are recombinant adenoviruses in which the specified genes are under control of the DF3/MUC1 tumor-selective promoter (6, 8). Adenoviral vectors were produced by homologous recombination in

the human embryonic kidney cell line 293 as described (3). Large scale production of recombinant adenovirus was accomplished by growth in 293 cells and purification by double cesium gradient ultracentrifugation as described (3). Titers of purified adenovirus were determined by spectrophotometry and by plaque assays.

Adenovirus infection. Cells suspended at 0.5 to 2 x 10^6/ml culture medium were infected with adenoviruses at a multiplicity of infection (MOI) of 1 to 1000 for 2 h, washed and then resuspended in fresh media. Cells were evaluated for the expression of the transgene at 24 to 48 h post infection.

Assay for luciferase activity. Luciferase activity was measured with D-luciferin (Analytical Luminescence Laboratory, San Diegi, CA) using a luminometer. Activity is presented as relative luminescent units (RLU) in an indicated number of cells.

Assays for β-galactosidase. (i) Chemiluminescence assay. Quantitation of enzyme activity was determined by a chemiluminescence assay using Galacto-Light system (Tropix, Inc., Bedford, MA) that detects 2 fg to 20 ng of β-galactosidase (9). Activity is presented as relative luminescent units (RLU) in an indicated number of cells. ii) Histochemical staining. Cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ for 10 min, rinsed with PBS, and then incubated with X-Gal (1 mg/ml), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS for 4 h. (iii) FACS-GAL assay (10). Briefly, 0.5-1 x 10^6 cells were suspended in 50 µl of serum-free culture medium at 37°C. An equal volume of 2 mM fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was added to

each aliquot of cells. The cells and FDG were mixed rapidly and incubated for 1 min at 37°C. Thereafter, cells were washed once with 4 ml ice-cold PBS and maintained in ice-cold PBS until analysis.

Tumor cell clonogenic assay. At 24 h after adenovirus infection, ganciclovir (GCV) was added to cells and incubated for 24 h. Serial dilutions of cells were plated on 30-mm culture dishes. Cells were incubated for 2 wks, and colonies (>50 cells) were stained with crystal violet and counted. Results are expressed as the surviving cell fraction ± SEM for the treated groups compared to controls.

Hematopoietic progenitor cell assays. Erythroid burst-forming units (BFU-E) and granulocyte-monocyte colony-forming units (CFU-GM) were assayed in a methylcellulose culture system (Stem Cell Technologies, Vancouver, British Columbia, Canada) containing recombinant human stem cell factor (50 ng/ml), GM-CSF (10 ng/ml), IL-3 (10 ng/ml), and erythropoietin (EPO) (3 U/ml). The numbers of colonies were counted after two weeks. For more primitive progenitor cells, the number of long-term culture-initiating cells (LTC-ICs) were determined by culturing serial dilutions of $CD34^+$ cells on irradiated bone marrow stromal cells in 96-well plates for 5 weeks. The number of wells that contained colonies was then assessed by growth in methylcellulose culture (Stem Cell Technologies) (4). The frequency of LTC-ICs was calculated by plotting the input cell number against the proportion of negative wells as described (4, 11).

Polymerase chain reaction (PCR) analysis. CD34+ cells,

CFU-GM and BFU-E picked from methylcellulose culture were digested at 56°C for 1-2 h with proteinase K (2 mg/ml) in cell lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin (Sigma), 0.45% NP40, and 0.45% Tween 20, as described (12). Samples were then heated at 95°C for 5 min. DNA was amplified using the GeneAmp PCR reagent kit (Perkin Elmer/Cetus, Norwalk, CT). The β-actin gene was used as an internal control and amplified using the primers 5'TCACCCACACTGTGCCCAT3' and 5'GCATTTGCGGTGGACGATG3'. The adenovirus E1A gene was amplified using primers 5'ATTACCGAAGAAATGGCCGC3' and 5'CCCATTTAACACGCCATG3'. The adenovirus E2B gene was amplified using primers 5'TCGTTTCTCAGCAGCTGTTG3' and 5'CATCTGAACTCAAAGCGTGG3' as described (13).

Statistical analysis. Results are presented as means ± SEM. Data comparisons were made by ANOVA. Pairwise comparisons were made using Fisher's PLSD (14) with STATVIEW 4.0 software (Abacus Concepts, Inc., Berkley, CA).

3. Induction of antigen-specific antitumor immunity with adenoviral-transduced dendritic cells.

Cell culture. DC were isolated from bone marrow cultures as described (15). Briefly, bone marrow flushed from the long bones of C57Bl/6 mice was treated with ammonium chloride to lyse red cells. Lymphocytes, granulocytes and Ia+ cells were depleted by incubation with monoclonal antibodies (MAbs) 2.43 (anti-CD8; ATCC, Rockville, MD), GK1.5 (anti-CD4; ATCC), RA3-3A1/6.1 (anti-

B220/CD4SR; ATCC), B21.2 (anti-Ia; ATCC), RB6-85C (anti-Gr-1; Pharmingen, San Diego, CA) and rabbit complement. The cells were plated in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 10 mM HEPES (pH 7.4), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 500 U/ml recombinant murine GM-CSF (Boehringer-Mannheim). After 7 d, the tightly adherent monocytes were harvested for transduction, while the nonadherent and loosely adherent cells were collected and replated in 100-mm Petri dishes (8×10^6 cells/dish). The nonadherent cells were removed after 30 min by washing, and medium containing GM-CSF was added to the dish. The cells were incubated for 18-24 h and the floating DC population was then harvested for analysis and transduction.

Recombinant adenoviral infection. Ad.βgal and Ad.MUC1 are structurally similar replication-deficient recombinant adenoviruses in which the lacZ and DF3/MUC1 genes (16), respectively, are under control of the cytomegalovirus (CMV) immediate-early promoter and enhancer (6, 17). DC and monocytes were incubated with recombinant adenovirus at the indicated multiplicity of infection (MOI) for 6 h, washed and then cultured in medium containing GM-CSF.

Analysis of adenoviral-transduced DC. Cells were washed with PBS and incubated with MAb D19-2F3-2 (anti-βgal; Boehringer-Mannheim), DF3 (anti-MUC1), M1/42/3.9.8 (anti-MHC Class I; ATCC), M5/114 (anti-MHC Class II; ATCC), 16-10A1 (anti-B7-1; provided by Dr. Hans Reiser, Dana-Farber), GL1 (anti-B7-2; Pharmingen) or 3E2 (anti-ICAM; Pharmingen) for 30 min on ice. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-

conjugated anti-hamster, -rat or -mouse IgG for analysis by FACScan (Becton-Dickinson). For immunoperoxidase staining, the cells were centrifuged onto slides, incubated with MAb DF3 or MAb M5/114 and stained by the avidin-biotin complex method (Vector Laboratories, Inc., Burlingame, CA) (18).

Mixed lymphocyte reactions. Control and transduced DC were treated with 20 Gy ionizing radiation. The cells were incubated at varying ratios with syngeneic (C57Bl/6) or allogeneic (Balb/c) T cells in 96-well flat-bottomed plates for 4-5 d. The T cells were prepared by passing spleen suspensions through nylon wool columns, incubation for 90 min in culture dishes and collection of the nonadherent cells. Stimulation of T cells was assessed by pulsing with 1 μCi/well [^{3}H]thymidine (New England Nuclear) for 6 h and monitoring for tritium incorporation.

Immunoblot analysis. Lysates from control and Ad.MUC1-transduced DC were subjected to electrophoresis in 6% polyacrylamide gels and analysis for reactivity with MAbs DF3 and DF3-P as described (19).

Immunizations. C57Bl/6 mice were injected intravenously with 5 x 10^5 DC or monocytes on day 0 and again on day 10.

CTL assays. CTL activity was determined by the lactate dehydrogenase (LDH) release assay (CytoTox, Promega, Madison, WI) (20). Splenocytes isolated from mice were subjected to Ficoll density gradient centrifugation. The splenocytes were incubated with target cells at varying E:T ratios in V bottom microtitration plates (Nunc;, Roskilde, Denmark), centrifuged for 3 min at 1000g and incubated for 4 h at 37°C. At the end of coculturing, 50 μl supernatant were transferred to an assay plate and incubated with

50 µl of substrate mixture for 30 min at room temperature. Absorbance was determined at 490-429 nm by microplate reader (Model 3550, BIO-Rad Laboratories, CA). Killing of target cells by effectors was determined by the formula: Cytotoxicity (%)=100 x (experiment release - spontaneous release)/(maximum release - spontaneous release).

Antitumor activity. Mice were immunized twice (day 0 and 10) by intravenous injection of 5 x 10^5 DC or monocytes. On day 18, mice were challenged subcutaneously with 2 x 10^5 MC-38 cells that stably express DF3/MUC1 (16). Tumors >3m or greater in diameter as determined by vernier callipers were scored as positive.

4. Second generation DF3/MUC1 adenoviral vectors.

a) DF3 promoter/TET on/off plasmids.

Construction of DF3 promoter/TET on/off plasmids. The rtTA gene or the tTA gene, which when expressed activate transcription of the TRE, was prepared from pTet-on or pTet-off (CLONTECH) by isolating fragments generated by digestion with BamH1 and EcoRI. The rtTA gene or the tTA gene was ligated into the EcoRV site of pDF3-βgal by blunt end ligation. The resulting plasmids, pDF3-Tet on and pDF3-Tet off, were thus derived by replacing the lacZ gene with rtTa or tTa, respectively.

Construction of pCMV-Tet on and pCMV-Tet off plasmids. The pCMV-Tet on and the pCMV-Tet off plasmids were constructed by replacement of the lacZ gene in pCMV-β-gal with rtTA and tTA, respectively, as described above.

Construction of pTRE-β-gal and pTRE-tk plasmids. The Phcmv*-1 promoter, which contains the Tet-responsive element (TRE) upstream

of the minimal CMV promoter, was isolated from pTRE (CLONTECH) by digestion with EcoRI and XhoI. The Phcmv*-1 Tet-responsive promoter was then ligated into XhoI and SpeI digested pCMV-βgal or pCMV-tk by blunt end ligation.

b) DF3/MUC1 promoter replication competent adenovirus

pKSCMVE1 and pAdCLxB were kindly provided by Dr. Brough, GenVec. pKSCMVE1 contains the sequences which encode the E1 region. pAdCLxB contains adenovirus 5 (Ad5). pDF3/CMV, the plasmid for the replication competent adenovirus vector, was generated using a first set of PCR primers, A2s(475)XS (5'-GGACTAGTAAGCTTCTCGAGCCCGTGAGTTCCTCAAGAGG-3') and A2a(921)NS (5'-TCCCCCGGGCTAGCATCGATCACCTCCGGCACAA-3') and produced a 480 bp fragment. The 480 bp PCR fragment was digested with the SpeI and SmaI, and then was ligated into the SpeI and StuI digested pAdCLxB (plasmid#1). A second set of PCR primers, DF3.5' (5'-TCTAGACTAGTGGACCCTAGGGTTCATCGGAG-3') and DF3.3' (5'-AACTCGAGGATTCAGGCAGGCGCTGGCT-3') produced a 780 bp fragment which contains the DF3 promoter which was ligated into the SpeI and XhoI digested plasmid#1 (plasmid#2). The ClaI fragment from pKSCMVE1~5kb was ligated into ClaI digested plasmid#2 to yield pDF3/CMV. pDF3 was constructed by deleting a CMV promoter from pDF3/CMV digested with SpeI and BamHI, blunt ending with klenow and then ligation. pDF3/CMV-GFP was constructed by inserting the GFP cDNA into BamHI site.

Recombinant adenoviruses were produced by homologous recombination in the human embryonic kidney cell line 293(11). Three ug of plasmid pDF3/CMV-GFP, pDF3/CMV, or pDF3 were mixed

with 6ug of pJM17, precipitated with $CaCl_2$, and used to transfect 293 cells. Recombinant adenovirus was isolated from a single plaque and expanded in 293 cells. The viral DNA was purified and analyzed by polymerase chain reaction (PCR). Two pairs of primers DF3.5' and DF3.3', A2S(475)XS and A2A(921)NS, were used to amplify and verify the presence of the DF3 promoter (-725 to +31) and Ad2 E1A in the viral genome.

Virus was prepared by infecting 80 15-cm plates of 293 cells and harvesting the detached cells after 48 h. The virus remains associated with the cell. Cells were collected by centrifugation at 1200 RPM for 5 min at 4°C. The cells were resuspended in 10 ml of cold PBS (Ca2+ and Mg2+ free), and were lysed by three repetitions of freeze and thaw. Cells were collected by centrifugation at 3000 RPM for 10 min at 4°C. The supernatant was layered onto a CsCl gradient containing equal parts of 1.45 g/ml and 1.20 g/ml CsCl in PBS and centrifuged for 2 h at 35000 rpm at 12° C in a Beckman Ti41 rotor. The virus band was removed, rebanded in a preformed CsCl gradient by ultracentrifugation for 18 h, and dialyzed into cold 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ containing 10% glycerol.

RESULTS AND DISCUSSION

1. Breast cancer selective gene expression and therapy mediated by recombinant adenovirus containing the DF3/MUC1 promoter.

Selective expression of β-galactosidase in human cell lines in vitro. A replication defective recombinant adenovirus was constructed that contains the β-galactosidase gene under control of the DF3 promoter. Expression of β-galactosidase was evaluated in Ad.DF3-βgal-infected MCF-7, ZR-75-1, BT-20, and MDA-MB231 breast cancer cells. In addition, Hs578Bst, a myoepithelial cell line derived from normal breast tissue and T98G, a human glioblastoma cell line were used in these studies. Each cell line was infected with either Ad.DF3-βgal or Ad.CMV-βgal at a MOI of 50. Expression of β-galactosidase was observed in MCF-7, ZR-75-1, and BT-20 cells infected with Ad.DF3-βgal, whereas little if any β-galactosidase activity could be detected in similarly infected Hs578Bst, MDA-MB231 and T98G cells. In contrast, all of these cell lines showed strong expression of β-galactosidase when infected with Ad.CMV-βgal in which the reporter gene is under control of CMV early promoter and enhancer. MCF-7 cells infected with Ad.DF3-βgal at an MOI of 50 exhibited highest β-galactosidase activity at day 3 to day 7 post infection. Transgene expression gradually decreased to approximately 15% of maximum at 2 weeks post infection.

Expression of β-galactosidase in Ad.DF3-βgal infected cells correlates with the expression of DF3. To assess whether there is a correlation between DF3 expression and capability of these cells to express β-galactosidase after Ad.DF3-βgal infection, we examined

the presence of DF3 antigen in MCF-7, ZR-75-1, and MDA-MB231 cells by FACS analysis. MCF-7 and ZR-75-1 cells exhibited strong reactivity with MAb DF3, a monoclonal antibody against DF3 antigen, while little if any MAb DF3 binding was detectable with Hs578Bst cells. Using the FACS-GAL assay, all MCF-7 and ZR-75-1 cells infected with Ad.DF3-βgal appear to express β-galactosidase with an increase in mean fluorescent intensity of up to 35-fold. In contrast, there was little if any expression of β-galactosidase in Ad.DF3-βgal-infected Hs578Bst cells. These findings support a correlation between the presence of cellular DF3 antigen and expression of β-galactosidase in Ad.DF3-βgal-infected cells.

Ad.DF3-tk sensitizes DF3-positive MCF-7 and ZR-75-1 breast cancer cells to GCV in vitro. Given the finding that the DF3 promoter can direct selective expression of a reporter gene, we replaced the β-galactosidase gene in Ad.DF3-βgal with HSV-tk. To determine whether Ad.DF3-tk can confer sensitivity to GCV, MCF-7 and ZR-75-1 cells were transduced with Ad.DF3-tk at MOIs of 10 and 50. Ad.CMV-tk was used in order to assess HSV-tk gene expression under control of the different promoters. Infection with Ad.DF3-tk had little effect on cell viability. Moreover, Ad.DF3-tk transduction conferred sensitivity of both MCF-7 and ZR-75 cells to GCV, while nontransduced cells or cells transduced with Ad.CMV-βgal or Ad.DF3-βgal (data not shown) were insensitive to GCV. The degree of Ad.DF3-tk-mediated GCV sensitivity was comparable to that obtained with Ad.CMV-tk. In contrast, when Ad.DF3-tk and Ad.CMV-tk were used to infect DF3-negative Hs578Bst epithelial cells, only Ad.CMV-tk infected cells were sensitive to GCV.

In vivo targeted gene expression in human breast cancer xenografts. To ascertain whether Ad.DF3-βgal can confer selective expression of β-galactosidase in vivo, we injected 2-5 x 10^8 pfu of Ad.DF3-βgal into MCF-7 and ZR-75-1 cells grown as xenografts in athymic nude mice. Three days after adenovirus infection, the tumors were excised and assayed for β-galactosidase activity. Expression of β-galactosidase was distributed extensively along the needle tract of Ad.DF3-βgal injection (as indicated by the co-localized charcoal particles) in both MCF-7 and ZR-75-1 tumor nodules. Blue staining was detectable within the tumor mass, but not in the surrounding normal tissue. In contrast, when Ad.DF3-βgal was injected into DF3-negative MDA-MB231 tumors grown in nude mice, there was no detectable β-galactosidase staining along the needle tract. Intramuscular injections of Ad.DF3-βgal also resulted in no detectable staining of β-galactosidase, while similar injections of Ad.CMV-βgal into skeletal muscle lead to strong expression of β-galactosidase.

β-galactosidase expression after systemic administration of Ad.DF3-βgal and Ad.CMV-βgal. To further evaluate adenovirus-mediated gene transfer to different tissues in vivo, 2 x 10^9 pfu of either Ad.CMV-βgal or Ad.DF3-βgal was injected via tail vein. Mice were sacrificed 4 days after the injection and sections of the liver, spleen, and lung were stained for β-galactosidase activity. Systemic injection of Ad.CMV-βgal resulted in expression of β-galactosidase in the liver parenchyma, in the splenic red pulp, and diffusely in the lung. By contrast, there was no detectable β-galactosidase staining in these tissues in mice injected with Ad.DF3-βgal. Several foci of β-galactosidase staining was detected

in MCF-7 tumors of animals that received systemic Ad.DF3-βgal. However, i.p. injection of Ad.DF3-βgal into mice bearing intraperitoneal MCF-7 tumor was associated with extensive expression of β-galactosidase in the tumor.

Treatment of intraperitoneal breast tumor. In order to evaluate the therapeutic efficacy of Ad.DF3-tk in human DF3-positive breast cancer cells in vivo, nude mice were inoculated i.p. with MCF-7 cells. These mice developed tumor masses throughout the peritoneal cavity and 2-4 ml of bloody ascites that contained tumor cells. MCF-7 tumor bearing mice were treated with Ad.DF3-βgal + saline, Ad.DF3-βgal + GCV, Ad.DF3-tk + saline and Ad.DF3-tk + GCV. Adenoviruses were injected i.p. on day 4 after tumor inoculation. GCV or saline injections were then administered on day 7 afer tumor inoculation. Untreated mice and those treated with Ad.DF3-βgal or Ad.DF3-tk + saline developed multiple intraperitoneal tumor with bloody ascites. In contrast, there was no grossly identifiable tumor mass or only a few small tumor clusters with no apparent ascites in the Ad.DF3-tk/GCV-treated mice.

2. Selective transgene expression for detection and elimination of contaminating carcinoma cells in hematopoietic stem cell sources.

The efficiency of adenovirus-mediated reporter gene expression was first evaluated in hematopoietic cell preparations using Ad.CMV-Luc and Ad.CMV-βgal. Luciferase and β-galactosidase activities were low but detectable in unfractionated PB and BM mononuclear cells at MOIs of 10 and 100. By contrast, there was

little if any detectable reporter gene expression in these cells when using Ad.DF3-βgal. Similar studies in MCF-7 breast cancer cells demonstrated a marked increase in efficiency (5-6 orders of magnitude) of Ad.CMV-Luc and Ad.CMV-βgal-mediated reporter gene expression. Moreover, as previously demonstrated (6), β-galactosidase expression was readily detectable in MCF-7 cells transduced with Ad.DF3-βgal. These findings indicated that MCF-7 cells are transduced more efficiently than hematopoietic cells by adenoviral vectors and that the tumor-selective DF3/MUC1 promoter can confer even greater selectivity of transgene expression. Two-color FACS-analysis further indicated that monocytes and macrophages ($CD14^+$ cells) are the major cell types in PB that express β-galactosidase when infected with Ad.CMV-βgal and not with Ad.DF3-βgal, while T ($CD3^+$) and B ($CD19^+$) cells express little if any transgene with either vector.

To determine if adenovirus mediates transgene expression in $CD34^+$ hematopoietic stem and progenitor cells, we performed two-color FACs analysis of enriched $CD34^+$ cells infected with Ad.CMV-βgal. At a MOI of 100, less than 4% of $CD34^+$ cells expressed the transgene, while approximately 11% of the $CD34^+$ cells were βgal positive at a MOI of 1000. These findings indicated that transduction of $CD34^+$ cells is inefficient compared to that for MCF-7 cells. A sensitive chemiluminescent assay showed that there was little β-galactosidase expression when enriched $CD34^+$ cells were transduced with Ad.CMV-βgal (~2,000 RLU/10^4 cells at a MOI = 10) and no β-galactosidase expression with Ad.DF3-βgal. By contrast to the $CD34^+$ cells, bone marrow stromal cells were transduced efficiently by Ad.CMV-βgal (10^6 RLU/10^4 cells at a MOI =

10). Moreover, the stromal cells exhibited little β-galactosidase expression following transduction with Ad.DF3-βgal. To substantiate the relatively low infectability of CD34+ cells by adenovirus, we used PCR to determine the relative copy number of virus per cell after infection. Purified bright $CD34^+$ cells infected with adenovirus were obtained by fluorescence sorting and DNA was extracted for PCR analysis of adenoviral E2B sequences. There was no detectable adenovirus in 10^3 $CD34^+$ cells infected at a MOI of 10, while the E2B signal was readily apparent from 10^3 transduced MCF-7 cells. A low level signal was obtained when assaying 10^4 infected $CD34^+$ cells. By comparison with an adenovirus standard, we estimate that there are approximately 10 copies of virus per MCF-7 cell and less than 0.01 copy per $CD34^+$ cell when cells were infected with adenovirus at a MOI of 10.

Adenovirus infection is a two step process involving the initial attachment of adenoviral fiber protein to a relatively ubiquitously expressed, but yet unidentified, receptor and then internalization through interaction of the adenoviral penton base with αv integrins, particularly αvβ3 and αvβ5 heterodimers (21, 22). FACS analysis indicated that $CD34^+$ cells had no detectable αv subunits, αvβ3 or αvβ5. By contrast, αv subunits were strongly expressed on breast cancer, lung cancer, prostate cancer and glioblastoma cells. The tumor cells expressed αvβ5 at high levels and αvβ3 to a lesser extent. These results indicated that the low level of adenoviral-mediated transduction in $CD34^+$, as compared to carcinoma, cells is attributable at least in part to the absence of integrins that contribute to adenoviral internalization.

The finding that adenovirus preferentially transduces carcinoma, as compared to hematopoietic, cells suggested that adenoviral-mediated reporter gene expression could be used to detect contaminating cancer cells in PB and BM. To address this issue, MCF-7 cells were premixed with PB cells at ratios of $1:10^2$ to $2:10^6$. Reporter activity of Ad.CMV-Luc- and Ad.CMV-βgal-infected cell mixtures reflected the number of contaminating MCF-7 cells. The level of luciferase activity mediated by Ad.CMV-Luc infection was significantly increased at a ratio of ten MCF-7 cell/5 x 10^5 PB mononuclear cells. Higher ratios were associated with increases in reporter gene expression. Similar results were obtained with Ad.CMV-βgal. Studies performed with Ad.DF3-βgal demonstrated a lower background with uncontaminated PB mononuclear cells and enhanced sensitivity with detection of one MCF-7 cell/5 x 10^5 PB cells. Similar results were obtained with Ad.DF3-βgal when ZR-75-1 breast cancer cells were mixed with PB cells. Other studies were performed on BM cells that had been contaminated (0.1%) with DF3/MUC1-positive breast, lung, prostate, and ovarian cancer cells. The contaminated BM cells demonstrated a dramatic elevation in reporter activity when using AD.DF3/βgal. Furthermore, contamination of BM with increasing numbers of MCF-7 cells resulted in higher levels of Ad.DF3-βgal-mediated reporter gene expression, while there was no increase in β-galactosidase expression when the BM cells were contaminated with DF3/MUC1 negative T98G glioblastoma cells.

To extend the observation of selective adenoviral-mediated reporter gene expression, we explored other approaches for detection of contaminating carcinoma cells. BM mononuclear cells

with and without contaminating MCF-7 cells were infected with Ad.DF3-βgal and then visualized for X-gal staining. Using this approach, the MCF-7 cells could be readily identified by blue staining. The contaminating cells were also readily apparent by fluorescence microscopy after staining with the fluorescence substrate FDG. Cells that expressed β-galactosidase also reacted with MAb DF3 (data not shown), a monoclonal antibody that detects DF3/MUC1 (23). These findings indicated that histochemical, as well as biochemical, approaches can be used for detection of contaminating tumor cells by adenoviral-mediated reporter gene expression.

The selectivity of adenoviral-mediated gene transduction for contaminating tumor cells supported the possibility of using this approach to purge hematopoietic cell populations. Previous studies have documented the strategy of expressing the HSV-tk gene for selective killing by GCV (6). To exploit this strategy for purging, adenovirus carrying HSV-tk under control of the CMV or DF3/MUC1 promoters was used to transduce PB cells pre-mixed with tumor cells. As determined by clonogenic survival, infection at a MOI of 10 followed by GCV treatment (10 to 1000 μM) resulted in the elimination of over 6-logs of contaminating MCF-7 cells. Infection with Ad.DF3-tk at a MOI of 100 and then treatment with 100 μM GCV killed approximately 6-logs of cancer cells. In addition, this approach effectively eliminated other contaminating breast, prostate, lung and glioblastoma tumor cells pre-mixed with BM cells.

A potential adverse effect of ex vivo purging is toxicity to hematopoietic progenitor cells. We thus assessed the effects of

adenoviral infection and GCV treatment on CFU-GM and BFU-E. Infection with Ad.CMV-tk or Ad.DF3-tk at a MOI of 10 followed by GCV (100 μM) treatment had little effect on CFU-GM and BFU-E as compared to GCV alone (≤10% cytotoxicity). Adenovirus infection alone at a MOI of 10 had little if any effect on colony formation. At a MOI of 100, there was a 17-19% decrease in BFU-E and CFU-GM when Ad.CMV-tk and GCV were used, while there was less of an effect with Ad.DF3-tk and GCV. Limiting dilution assays were also performed on enriched $CD34^+$ cells to assess the effects of adenovirus and GCV treatment on long-term culture initiating cells (LTC-ICs). The results demonstrate that infection with Ad.CMV-tk with or without GCV treatment has little if any effect on the regeneration and differentiation of the primitive progenitor cells. Additional experiments were performed to determine if adenovirus is detectable in the progeny cells after adenoviral purging of progenitor cells. CFU-GMs and BFU-Es were picked from methylcellulose and cultured with 293 cells. No live adenovirus was rescued in three separate experiments. RT-PCR analysis of CFU-GM and BFU-E colonies failed to detect any transgene expression mediated by recombinant adenovirus. Importantly, the finding that PCR analysis did not detect the presence of adenoviral E1a sequences indicated no wild type adenovirus replication.

3. Induction of antigen-specific antitumor immunity with adenoviral-transduced dendritic cells.

Flow cytometry was used to define the phenotype of DC following transduction with recombinant adenovirus. DC derived

from bone marrow expressed MHC class I and II products, costimulatory molecules and ICAM-1 (15). Transduction with Ad.βgal resulted in a similar pattern of antigen expression. Moreover, transduction with Ad.MUC1 was associated with DF3/MUC1 expression and little if any effect on cell surface levels of MHC, costimulatory or adhesion molecules. The Ad.MUC1-transduced DC exhibited a typical morphology with veiled dendrites. Staining with MAb M5/114 (anti-MHC class II) and MAb DF3 demonstrated expression of DF3/MUC1 by the transduced DC. Immunoblot analysis of the Ad.MUC1 transduced DC confirmed DF3/MUC1 expression. Whereas MAb DF3 detects glycosylated MUC1, the finding that MAb DF3-P reacts with a ~55 kD protein in the transduced DC also provides support for detection of the unglycosylated protein core (19).

DC are potent stimulators of primary mixed lymphocyte reactions (MLR) (24, 25). To assess in part the function of Ad-transduced DC, we compared their effects in primary allogeneic MLR with that obtained from non-transduced DC. The results demonstrate that DC transduced with Ad.MUC1 or Ad.βgal at an MOI of 100 exhibit the same potent stimulatory function as control DC. By contrast, DC transduced at MOIs of 200 or 500 exhibited decreases in viability and in T cell stimulation. These results indicate that expression of adenoviral, rather than the transgene, proteins is responsible for the loss of DC function.

To determine whether Ad-transduced DC induce antitumor immunity, we immunized mice twice with uninfected DC, Ad.MUC1-transduced DC or Ad.βgal-transduced DC. Splenocytes were assayed for CTL activity using as targets syngeneic MC-38

carcinoma cells that stably express DF3/MUC1 (16). T cells from mice immunized with Ad.MUC1-transduced DC exhibited strong activity against MC-38/MUC1, but not wild-type MC-38, cells. CTLs from these mice also induced lysis of Ad.MUC1-, and not Ad.βgal-transduced, MC-38 cells. By contrast, T cells from mice immunized with Ad.βgal-transduced DC exhibited lysis of only the Ad.βgal-transduced MC-38 cells. These findings indicated that Ad-transduced DC induce immunity which is directed against the transgene.

Incubation of CTLs from mice immunized with Ad.MUC1-transduced DC with anti-CD4 or anti-CD8 antibodies blocked lysis of the MC-38/MUC1 targets. These results indicated that Ad.MUC1-transduced DC generate MHC class I and II-restricted T cell responses. The finding that incubation of MC-38/MUC1 targets with MAb DF3 blocks lysis provided further support for specificity against DF3/MUC1. Moreover, incubation of YAC-1 cells with the CTLs showed no specific lysis. Immunization of mice with Ad.MUC1-transduced DC also inhibited growth of MC-38/MUC1 tumors, while Ad.βgal-transduced or non-transduced DC had no effect on tumor growth. To assess the potency of the Ad-transduced DC, we compared induction of immunity with that obtained when using monocytes. The efficiency of Ad.βgal- and Ad.MUC1-mediated transduction of DC and monocytes was similar. However, immunization with Ad.MUC1-transduced monocytes was less effective than Ad.MUC1-transduced DC in the induction of CTL activity. Ad.MUC1-transduced monocytes were also less effective than the transduced DC in inhibiting the growth of MC38/MUC1 tumors. These

findings are in concert with the greater potency of DC as APCs (26).

Previous studies have demonstrated retroviral transduction of human $CD34^+$ progenitor cells and then differentiation of the transduced cells into DC by cytokine stimulation (27, 28). The differentiated DC expressed the transgene and were functional in stimulating T cells in vitro (27, 28). Whereas retroviral transduction requires proliferating cells, adenoviral-transduced gene expression is not dependent on cell growth. The present studies demonstrate that murine DC can be efficiently transduced by adenoviral vectors. Transduction of DC with Ad.MUC1 or Ad.βgal at an MOI of 100 resulted in over 80% of the cells expressing the transgene. Similar transduction efficiencies were obtained in monocytes and fibroblasts. Whereas transduction at an MOI of 100 had no effect on stimulation in the MLR assay, higher MOIs (200 and 500) resulted in lower levels of T cell proliferation. This finding was associated with cytopathic effects observed at the higher MOIs.

Studies with retrovirally transduced $CD34^+$ cells that differentiate to DC have not been performed in an animal model; therefore, it is not known whether these cells are useful for in vivo immunization. The present studies demonstrate that immunization with Ad.MUC1- or Ad.βgal-transduced mouse DC induce CTL responses that are specific for the transgene. Reactivity against adenoviral antigens was apparently low based on the selectivity of the CTL response against DF3/MUC1 or βgal. Treatment of the CTLs with antibodies against T cell subsets

indicated that the Ad-transduced DC stimulate a $CD4^+$ and $CD8^+$ immune response.

4. Second generation DF3/MUC1 adenoviral vectors.

a) Tet on/off system.

We constructed recombinant adenoviruses expressing the Tet-on/off system based on the hypothesis that the Tet on/off regulator and TRE response cassettes should enhance transcription off the DF3/MUC1 promoter (Fig. 1). After we we transfected with Tet-on/off regulator plasmid into MCF-7 cell lines by calcium phosphate method, we analyzed tTA expression by Western blotting using the Tet R monoclonal antibody. tTA protein was not detected (Table 1). In addition after infection with recombinant adenovirus bearing the Tet-on/off regulator gene, we detected tTA protein weakly in only the positive control sample (Table 2). These findings indicated that expression of tTA protein is weak or the Tet-on/off regulator is not functioning properly.

Figure 1

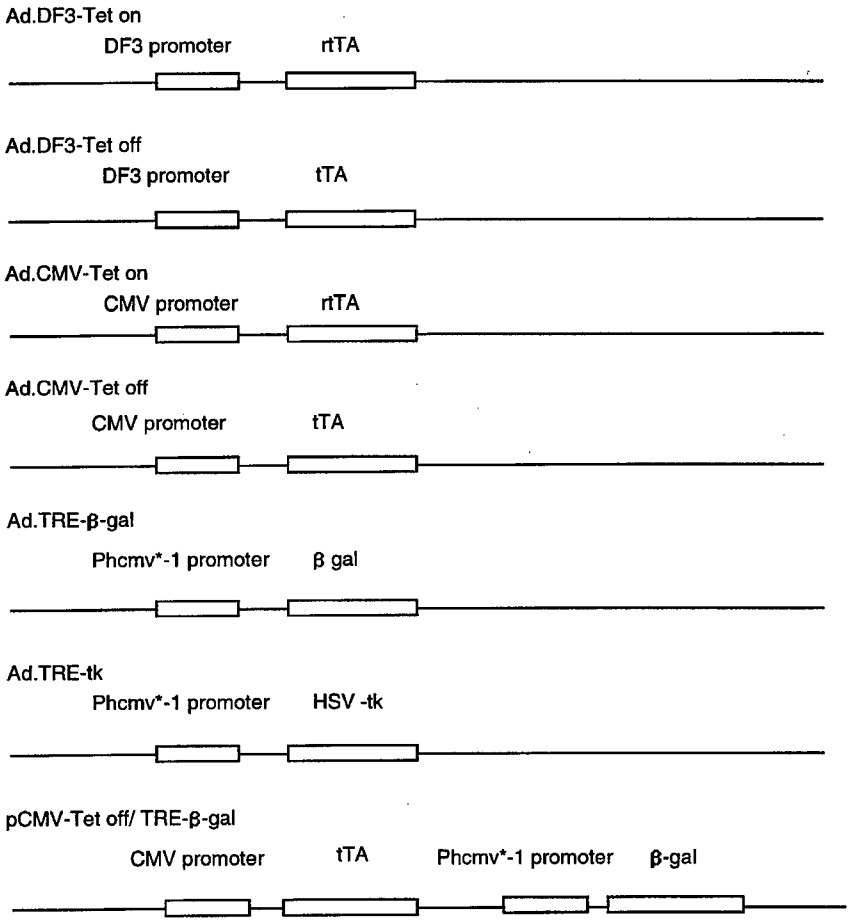


Table 1

Transfection with pCMV-TET on/off into MCF 7 cell lines

	positive control	pCMV on Tc +	pCMV off Tc -	negative control
actual result	-	-	-	-
expected result	+	+	+	-

*positive control; CHO-AA8 Tet off cell line
negative control; no plasmid

Table 2

Infection with Ad.CMV-TET on/off into MCF-7 cell lines

	positive control	Ad.CMV on Tc +	Ad.CMV off Tc -	negative control
actual result	+	-	-	-
expected result	+	+	+	-

*positive control;CHO-AA8 Tet off cell line
negative control; no adenovirus infection

Next, we transfected the pTRE response plasmid containing the LacZ gene, pTRE-β-gal, into the CHO-AA8 Tet off cell line. This cell line stably expresses the tTA protein in the Tet-off system. We stained these cells with X-gal (Table 3).

Table 3

Transfection with pTRE-β-gal into the CHO-AA8 Tet off cell lines

	positive control pCMV-β-gal	pTRE-β-gal Tc -	negative control
actual result	+	+	-
expected result	+	+	-

After infection with the TRE response recombinant adenovirus, Ad.TRE-βgal, into the same cell lines, we again assayed for X-gal staining. The TRE response cassettes induced the LacZ gene (Table

4); however, the efficiency was low. To determine whether the Tet-on/off regulator and TRE response cassettes function properly, we coinfected MCF-7 cells with recombinant adenoviruses expressing the Tet on/off regulator and TRE response gene. The results indicate that the Tet on/off regulator cassette is not functional (Table 5). A second problem is that the TRE response plasmid works properly, but the TRE response recombinant adenovirus is not functional. Whereas it is difficult to infect with two adenoviruses because of virus interference, we attempted and succeeded to construct a plasmid with the Tet on/off regulator and TRE response cassettes, pCMV-Tet off/TRE-β-gal (Fig. 1). The construction was accurate as determined by digestion with restriction enzymes and PCR analysis of the Tet off regulator and TRE response sequences. LacZ gene expression was analyzed under Tet off regulator cassettes by X-gal staining after the transfection of this plasmid into 293 cell lines (Table 6). Although cells were strongly positive when transfected with pCMV-β-gal as a positive control, only a few cells were positive when we transfected the pCMV-Tet off/TRE-β-gal in the absence of Tet. These findings indicate that the efficiency of transduction of this plasmid is low or that this plasmid is not functional under the Tet off regulator cassette. Moreover, studies with Dox indicated that LacZ gene expression is not under control of the Tet off regulator and/or TRE response cassettes (Table 7). Finally, the results suggest that LacZ gene expression in our plasmids is regulated by the CMV promoter in Tet off regulator (Table 7).

Table 4

Infection with Ad.TRE-β-gal into CHO-AA8 Tet off cell lines

	positive control pCMV-β-gal	Ad.TRE-β-gal Tc -	Ad.TRE-β-gal Tc +	negative control
actual result	95%	3%	0%	NT
expected result	+	+	-	-

Table 5

β-gal staining to examine that the Tet on/off regulator and TRE response cassettes function properly (Coinfection with Tet on/off regulator and TRE response adenoviruses at the same time into MCF-7 cells)

	positive control Ad.CMV-β-gal	Ad.CMV on +Ad.TRE-β-gal Tc +	Ad.CMV on +Ad.TRE-β-gal Tc -	Ad.CMV off +Ad.TRE-β-gal Tc +	Ad.CMV off +Ad.TRE-β-gal Tc -	negative control
actual result	+	-	-	-	-	NT
expected result	+	+	-	-	+	-

Table 6

β-gal staining after transfection with pCMV-Tet off/TRE-β-gal into 293 cell lines

	positive control pCMV β gal	pCMV-Tet off/TRE-β-gal Tc -	Tc +	negative control
actual result	++	±	±	-
expected result	++	+~++	-	-

Table 7

β-gal staining after transfection with pCMV-Tet off/β-gal into 293 cell line and exposure to various Dox concentrations (ng/ml)

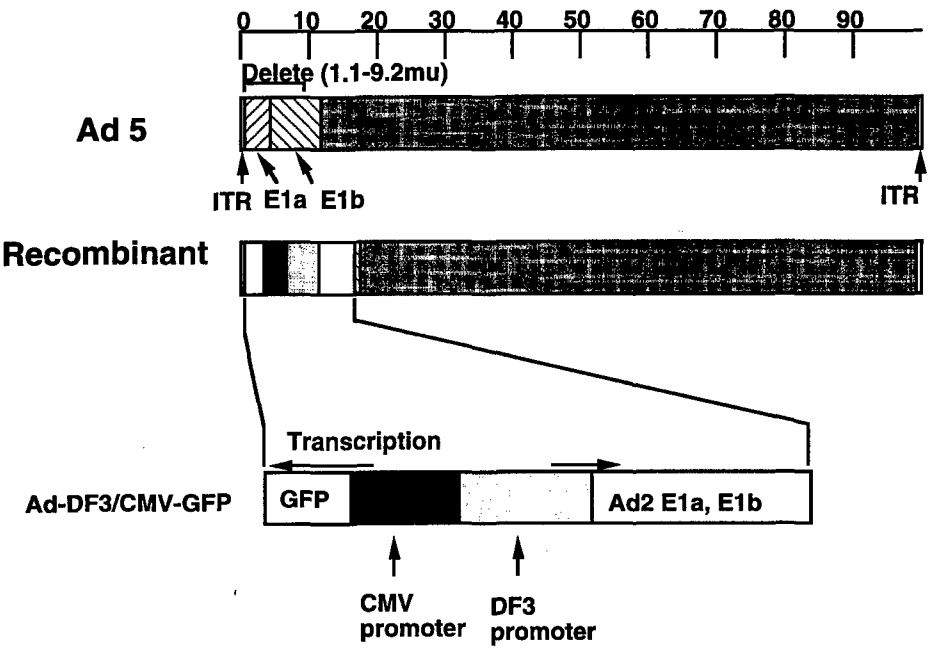
	positive control	Dox.(0)	Dox.(10)	Dox.(100)	Dox.(1000)	negative control
actual result	++	+	±	-	±	-
expected result	++	+~++	-	-	-	-

Collectively, these results demonstrate that we have been unsucsessful in developing the Tet on/off system as an enhance for the CMV and DF3/MUC1 promoters.

b) DF3/MUC1 promoter-replication competent adenoviral vector

The difficulties encountered in generating a DF3/MUC1 promoter vector that is enhanced by the Tet on/off system prompted the development of a replication competent adenovirus that is selective for DF3/MUC1-positive tumor cells. In this strategy, enhancement of the DF3/MUC1 promoter is not necessary because the adenovirus is capable of replicating in DF3/MUC1-positive cells. Replication of the adenovirus alone could be sufficient to induce cell killing. Moreover, expression of a suicide gene in the DF3/MUC1 promoter-replication competent adenovirus increases killing of the DF3/MUC1-positive tumor cells. To design such a vector, we have placed E1a and E1b control of the DF3/MUC1 promoter. In the opposite direction, the CMV promoter drives a transgene which, in the inital construction is the green fluorescence protein (GFP), but in second generation vectors will be a suicide gene (Fig. 2). The initial vector construct has been completed and we have produced the adenovirus. In vitro studies are underway which will determine if these adenoviruses are selectively competent for replication in DF3/MUC1 positive tumor cells.

Figure 2. DF3 promoter replication-competent adenovirus



CONCLUSIONS

1. Breast cancer selective gene expression and therapy mediated by recombinant adenoviruses containing the DF3/MUC1 promoter.

These results suggest that recombinant adenoviruses containing the DF3/MUC1 promoter and therapeutic genes could have applicability in gene therapy of breast cancer. Recombinant adenoviral vectors containing the DF3/MUC1 promoter and other candidate therapeutic genes including cytochrome P450, and cytosine deaminase have also been developed. However, E1-deleted adenoviral vectors used in our studies are "first generation" vectors. To achieve the requisite level of expression of the transgene and selectivity for breast tumors, additional vectors will need to be developed that incorporate of the DF3/MUC1 promoter. In this context, enhanced expression of the DF3/MUC1 promoter-driven transgenes may eventually lead to a vector system that is practical for human gene therapy.

2. Selective transgene expression for detection and elimination of contaminating carcinoma cells in hematopoietic stem cell sources.

These studies demonstrate that replication defective adenoviral vectors containing the DF3/MUC1 carcinoma-selective promoter can be used to selectively transduce contaminating carcinoma cells in PB and BM. The sensitivity of this approach will need to be compared directly with that of other techniques currently being used in clinical setting. However, our findings suggest that the use of adenoviral vectors for detection and

purging of contaminating breast cancer cells may be superior to those currently available. This approach is now being applied in clinical studies.

3. Induction of antigen-specific antitumor immunity with adenoviral-transduced dendritic cells.

Induction of anti-DF3/MUC1 immunity with the Ad.MUC1-transduced DC was sufficient to specifically inhibit the growth of DF3/MUC1-positive tumor cells. These findings support the usefulness of Ad-transduced DC for in vivo immunization against tumor-associated antigens. Moreover, the results of these studies have provided the experimental basis for immunizing with Ad.MUC1 transduced DC in the treatment of human breast cancer.

4. Second generation DF3/MUC1 adenoviral vectors.

We have developed adenoviral vectors that utilize the DF3/MUC1 promoter to selectively drive exogenous genes in human breast cancer cells. The initial studies in an animal model of a human breast tumor xenograft demonstrated that enhancement of the DF3/MUC1 promoter activity would be needed to achieve more substantial responses in the clinical setting. To this end, we attempted to constuct vectors with the DF3/MUC1 promoter that were enhanced by the more potent and selectable Tet on/off system. The complexity of the construction precluded the development of a functional vector. Consequently, we have instead developed an adenovirus that is replication competent in DF3/MUC1-positive cells. The DF3/MUC1 promoter-replication competent virus is designed to replicate in MUC1-positive cells and thereby selectively express a suicide gene that will kill those cells.

The DF3/MUC1 promoter-replication competent virus also obviates the need to enhance activity of the DF3/MUC1 promoter because this strategy has the advantage of viral replication in the DF3/MUC1-positive cells. This vector system will be developed for potential applicability in the gene therapy of human breast cancer.

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Selective Transgene Expression for Detection and Elimination of Contaminating Carcinoma Cells in Hematopoietic Stem Cell Sources

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Abstract

Tumor contamination of bone marrow (BM) and peripheral blood (PB) may affect the outcome of patients receiving high dose chemotherapy with autologous transplantation of hematopoietic stem cell products. In this report, we demonstrate that replication defective adenoviral vectors containing the cytomegalovirus (CMV) or DF3/MUC1 carcinoma-selective promoter can be used to selectively transduce contaminating carcinoma cells. Adenoviral-mediated reporter gene expression in breast cancer cells was five orders of magnitude higher than that found in BM, PB, and $CD34^+$ cells. Our results demonstrate that $CD34^+$ cells have low to undetectable levels of integrins responsible for adenoviral internalization. We show that adenoviral-mediated transduction of a reporter gene can detect one breast cancer cell in 5×10^5 BM or PB cells with a vector containing the DF3/MUC1 promoter. We also show that transduction of the *HSV-tk* gene for selective killing by ganciclovir can be exploited for purging cancer cells from hematopoietic stem cell populations. The selective expression of TK followed by ganciclovir treatment resulted in the elimination of 6-logs of contaminating cancer cells. By contrast, there was little effect on CFU-GM and BFU-E formulation or on long term culture initiating cells. These results indicate that adenoviral vectors with a tumor-selective promoter provide a highly efficient and effective approach for the detection and purging of carcinoma cells in hematopoietic stem cell preparations. (*J. Clin. Invest.* 1996. 98:2539–2548.) Key words: adenovirus • bone marrow • breast cancer • thymidine kinase • gene therapy

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1. *Abbreviations used in this paper:* Ad, adenovirus; β-gal, β-galactosidase; BFU-E, erythroid burst-forming unit; BM, bone marrow; CFU-GM, granulocyte-macrophage colony-forming unit; CMV, cytomegalovirus; FDG, fluorescein di-β-D-galactopyranoside; GCV, ganciclovir; HSV-tk, herpes simplex virus thymidine kinase; LTC-ICs, long-term culture-initiating cells; MOI, multiplicity of infection; PB, peripheral blood; RLU, relative luminescent units; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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Introduction

High dose chemotherapy followed by autologous transplantation of bone marrow (BM)[1] or peripheral blood (PB) as sources of hematopoietic stem cells is being used as a treatment option for patients with breast cancer (1–4). While this approach results in a proportion of patients with prolonged disease-free survival, most patients eventually relapse. One potential explanation for relapse is reinfusion of tumor cells that contaminate the hematopoietic cell preparations (5–7). Immunocytochemistry (5, 8), flow cytometry (8, 9), and PCR analysis (10, 11) have been used to detect contaminating breast cancer cells in BM and PB preparations. Although the significance of breast cancer cell contamination to relapse remains unclear, tumor-free hematopoietic stem cell products for autologous transplantation are nonetheless desirable. In this context, various approaches using mAbs or cytotoxic drugs have been developed for purging of carcinoma cells from BM or PB collections (12–17). These approaches have resulted in the elimination of two to five logs of clonogenic breast cancer cells and varying degrees of toxicity to hematopoietic progenitor and stem cells.

Gene therapy is a potentially novel approach for the purging of carcinoma cells from hematopoietic stem cell preparations. However, efficacy of purging cancer cells will require gene delivery systems which possess a high gene transduction efficiency and target cell specificity. Human adenoviruses are nonenveloped double-stranded DNA viruses which when deleted at the E1 region are replication defective (18). Adenovirus-mediated gene transfer is a highly efficient means of delivering genetic material into a wide spectrum of cells in vitro and in animals. However, in the setting of bone marrow purging, one goal is the selective transduction of exogenous genes into contaminating cancer cells. A potential strategy to achieve such selectivity would be to use a tumor cell specific/selective promoter to direct the expression of a therapeutic gene in the desired target cell. In this context, recent studies have demonstrated that the promoter of the DF3/MUC1 gene can be used to confer selective expression of heterologous genes in breast cancer cells (19, 20). DF3/MUC1 antigen is a member of a family of high molecular weight glycoproteins which are aberrantly overexpressed in breast and other carcinomas (21–23). Adenoviral vectors containing the β-galactosidase or the herpes simplex virus thymidine kinase (*HSV-tk*) gene under control of the DF3 promoter have thus been developed to confer efficient and selective expression of these genes in cancer cells (20).

In the present work, we demonstrate that adenoviral vectors containing the DF3/MUC1 promoter can be used for detection of carcinoma cells in preparations of hematopoietic stem cell sources. The results also demonstrate that selective expression of therapeutic genes in contaminating cancer cells

is an efficient approach for purging of hematopoietic stem and progenitor cells.

Methods

Cell lines. The MCF-7, ZR-75-1, BT-20, and SKBR3 breast carcinoma, the A549 lung carcinoma, DU145 prostate carcinoma, SKOV3 ovarian carcinoma, and T98G human glioblastoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). Cells were grown as monolayers in recommended culture medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Human hematopoietic cells. Human PB mononuclear cells were isolated by Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) density gradient centrifugation (d = 1.077, 400 g) from leukocyte-enriched leukopaks of healthy donors. Cells were suspended in RPMI-1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Bone marrow was obtained from filters used to prepare harvested marrow from normal donors and the mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation. Bone marrow stromal cells were isolated by adherence (24).

$CD34^+$ cells were isolated using the Ceprate LC cell separation system (CellPro Inc., Bothell, WA). In brief, BM cells were incubated with a biotinylated mouse anti-$CD34^+$ mAb, washed and then passed through an avidin column. Nonadsorbed cells were removed by washing, and adsorbed cells were eluted from the column. The enriched cells (80–90% $CD34^+$) were maintained in Iscove's MEM containing 12.5% FBS, 12.5% horse serum, and 1 μM hydrocortisone.

Antibody reaction and FACS® analysis. mAbs used were specifically reactive with the cell surface antigens: CD3 (T3, Coulter Immunology, Miami, FL), CD13 (L138, Becton Dickinson, San Jose, CA), CD19 (B4, Coulter Immunology), CD34 (Becton Dickinson), CD51 (integrin αv, clone 1980; Chemicon Inc., Temecula, CA), integrin αvβ3 (LM609, kindly provided by Dr. David Cheresh, Scripps Research Institute, La Jolla, CA) and integrin αvβ5 (25) (clone B5-IA9, generously provided by Dr. Martin E. Hemler, Dana-Farber Cancer Institute, Boston, MA). Cells were incubated with antibody for 30 min on ice. If the antibody was not directly conjugated with FITC or phycoerythrin (PE), a secondary antibody conjugated with FITC or PE (Sigma Chemical Co., St. Louis, MO) was used for indirect fluorescence labeling. Cells were then washed and evaluated by flow cytometric analysis.

Recombinant adenoviruses (Ad). Ad.CMV-βgal, Ad.CMV-tk (20), and Ad.CMV-Luc (kindly provided by Dr. Robert Gerard, University of Texas, Austin, TX) (26) are replication-deficient recombinant adenoviruses in which the luciferase, β-galactosidase, and *HSV-tk* genes, respectively, are under control of the cytomegalovirus (CMV) immediate-early promoter and enhancer. Ad.DF3-βgal and Ad.DF3-tk are recombinant adenoviruses in which the specified genes are under control of the DF3/MUC1 tumor-selective promoter (20, 27). Adenoviral vectors were produced by homologous recombination in the human embryonic kidney cell line 293 as described (28). Large scale production of recombinant adenovirus was accomplished by growth in 293 cells and purification by double cesium gradient ultracentrifugation as described (28). Titers of purified adenovirus were determined by spectrophotometry and by plaque assays.

Adenovirus infection. Cells suspended at 0.5 to 2.0 × 10^6/ml culture medium were infected with adenoviruses at a multiplicity of infection (MOI) of 1 to 1,000 for 2 h, washed, and then resuspended in fresh media. Cells were evaluated for the expression of the transgene at 24 to 48 h after infection.

Assay for luciferase activity. Luciferase activity was measured with D-luciferin (Analytical Luminescence Laboratory, San Diego, CA) using a luminometer. Activity is presented as relative luminescent units (RLU) in an indicated number of cells.

Assays for β-galactosidase. (*i*) Chemiluminescence assay: quantitation of enzyme activity was determined by a chemiluminescence assay using Galacto-Light system (Tropix, Inc., Bedford, MA) that detects 2 fg to 20 ng of β-galactosidase (29). Activity is presented as RLU in an indicated number of cells. (*ii*) Histochemical staining; cells were fixed with 0.5% glutaraldehyde in PBS containing 1 mM $MgCl_2$ for 10 min, rinsed with PBS, and then incubated with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (1 mg/ml), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS for 4 h. (*iii*) FACS®-GAL assay (30): briefly, 0.5–1.0 × 10^6 cells were suspended in 50 μl of serum-free culture medium at 37°C. An equal volume of 2 mM fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was added to each aliquot of cells. The cells and FDG were mixed rapidly and incubated for 1 min at 37°C. Thereafter, cells were washed once with 4 ml ice-cold PBS and maintained in ice-cold PBS until analysis.

Tumor cell clonogenic assay. At 24 h after adenovirus infection, ganciclovir (GCV) was added to cells and incubated for 24 h. Serial dilutions of cells were plated on 30-mm culture dishes. Cells were incubated for 2 wk, and colonies (> 50 cells) were stained with crystal violet and counted. Results are expressed as the surviving cell fraction±SEM for the treated groups compared to controls.

Hematopoietic progenitor cell assays. Erythroid burst-forming units (BFU-E) and granulocyte-monocyte colony-forming units (CFU-GM) were assayed in a methylcellulose culture system (Stem Cell Technologies, Vancouver, Canada) containing recombinant human stem cell factor (50 ng/ml), GM-CSF (10 ng/ml), IL-3 (10 ng/ml), and erythropoietin (EPO) (3 U/ml). The numbers of colonies were counted after 2 wk. For more primitive progenitor cells, the number of long-term culture-initiating cells (LTC-ICs) were determined by culturing serial dilutions of $CD34^+$ cells on irradiated bone marrow stromal cells in 96-well plates for 5 wk. The number of wells that contained colonies was then assessed by growth in methylcellulose culture (Stem Cell Technologies) (24). The frequency of LTC-ICs was calculated by plotting the input cell number against the proportion of negative wells as described (24, 31).

PCR analysis. $CD34^+$ cells, CFU-GM, and BFU-E picked from methylcellulose culture were digested at 56°C for 1–2 h with proteinase K (2 mg/ml) in cell lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.1 mg/ml gelatin (Sigma Chemical Co.), 0.45% NP-40, and 0.45% Tween 20, as described (32). Samples were then heated at 95°C for 5 min. DNA was amplified using the GeneAmp PCR reagent kit (Perkin Elmer/Cetus Corp., Norwalk, CT). The β-actin gene was used as an internal control and amplified using the primers 5'TCACCCACACTGTGCCCAT3' and 5'GCATTTGCGGTGGACGATG3'. The adenovirus *E1A* gene was amplified using primers 5'ATTACCGAAGAAATGGCCGC3' and 5'CCCATTTAACACGCCATG3'. The adenovirus *E2B* gene was amplified using primers 5'TCGTTTCTCAGCAGCTGTTG3' and 5'CATCTGAACTCAAAGCGTGG3' as described (33).

Statistical analysis. Results are presented as means±SEM. Data comparisons were made by ANOVA. Pairwise comparisons were made using Fisher's PLSD (34) with STATVIEW 4.0 software (Abacus Concepts, Inc., Berkeley, CA).

Results

The efficiency of adenovirus-mediated reporter gene expression was first evaluated in hematopoietic cell preparations using Ad.CMV-Luc and Ad.CMV-βgal. Luciferase and β-galactosidase activities were low but detectable in unfractionated PB and BM mononuclear cells at MOIs of 10 and 100 (Fig. 1 *A*). By contrast, there was little if any detectable reporter gene expression in these cells when using Ad.DF3-βgal (Fig. 1 *A*). Similar studies in MCF-7 breast cancer cells demonstrated a marked increase in efficiency (five orders of magnitude) of Ad.CMV-Luc and Ad.CMV-βgal-mediated reporter gene expression (Fig. 1 *B*). Moreover, as previously demon-

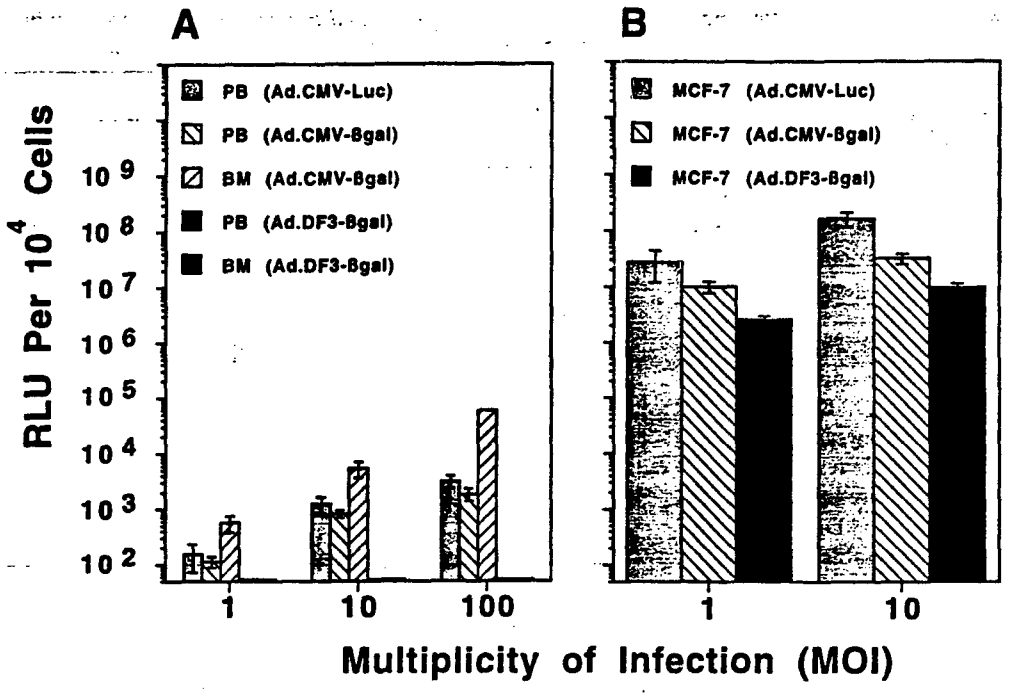
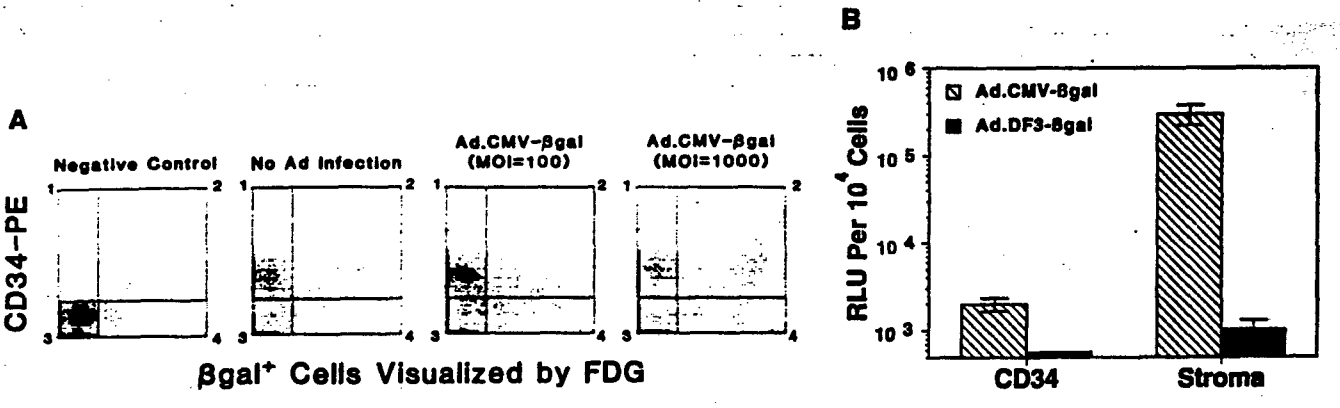


Figure 1. Analysis of adenovirus-mediated reporter gene expression in PB, BM, and breast cancer cells. (*A*) PB and BM mononuclear cells. (*B*) MCF-7 breast cancer cells. Cells were infected with Ad.CMV-Luc, Ad.CMV-βgal, or Ad.DF3-βgal at the indicated MOIs for 2 h at 37°C, washed, and cultured for 48 h. Cells were then lysed and assayed for luciferase or β-galactosidase activity. The results are presented as relative luminescent units (*RLU*) in the indicated number of cells (mean±SEM). Results were obtained from four to nine experiments.

strated (20), β-galactosidase expression was readily detectable in MCF-7 cells transduced with Ad.DF3-βgal (Fig. 1 *B*). These findings indicated that MCF-7 cells are transduced more efficiently than hematopoietic cells by adenoviral vectors and that the tumor-selective DF3/MUC1 promoter can confer even greater selectivity of transgene expression. Two-color FACS®-analysis further indicated that monocytes and macrophages (CD14⁺ cells) are the major cell types in PB that express β-galactosidase when infected with Ad.CMV-βgal and not with Ad.DF3-βgal, while T (CD3⁺) and B (CD19⁺) cells



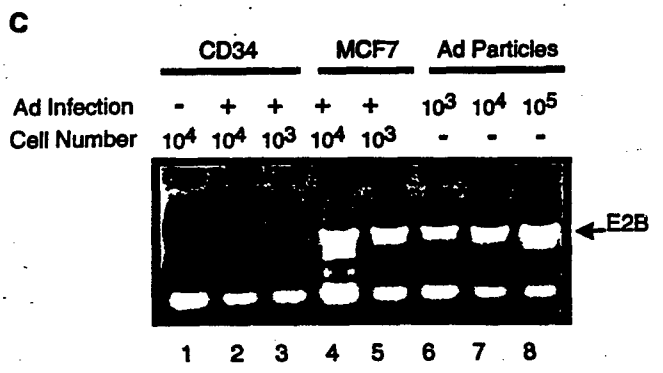


Figure 2. Analysis of adenovirus-mediated gene transfer in CD34⁺ and bone marrow stromal cells. (*A*) Two-color FACS analysis to detect β-galactosidase activity in enriched CD34⁺ cells infected with Ad.CMV-βgal or Ad.DF3-βgal. At 24 h after infection, CD34⁺ cells were stained with PE-conjugated anti-CD34 mAb. Cells expressing β-galactosidase were visualized by the fluorescence substrate, FDG. (*B*) β-galactosidase activity in enriched CD34 cells and bone marrow stromal cells infected with Ad.CMV-βgal or Ad.DF3-βgal. Cells were infected at a multiplicity of infection (*MOI*) of 10 for 2 h at 37°C, washed, and cultured for 48 h. β-galactosidase activity was measured by a luminescence assay. The results are presented as relative luminescent units (*RLU*) in the indicated number of cells (mean±SEM for three samples). (*C*) Determination of adenoviral DNA in adenovirus-infected CD34⁺ and MCF-7 cells. Cells were infected with adenovirus (MOI = 10) at 37°C for 2 h. Cells were incubated with trypsin/EDTA solution at 37°C for 5 min and washed three times with medium. DNA extracted from 10^4 (lanes *2* and *4*) and 10^3 (lanes *3* and *5*) fluorescence-sorted CD34⁺ cells or MCF-7 cells infected with adenovirus were used for PCR amplification (25 cycles) of a 0.86 kb sequence in the adenoviral E2B gene. Adenoviral DNA equivalent to 10^3, 10^4, and 10^5 pfu was used as a reference control.

express little if any transgene with either vector (data not shown).

To determine if adenovirus mediates transgene expression in $CD34^+$ hematopoietic stem and progenitor cells, we performed two-color FACS® analysis of enriched $CD34^+$ cells infected with Ad.CMV-βgal. At a MOI of 100, < 4% of $CD34^+$ cells expressed the transgene, while ~ 11% of the $CD34^+$ cells were βgal positive at a MOI of 1,000 (Fig. 2 *A*). These findings indicated that transduction of $CD34^+$ cells is inefficient compared with that for MCF-7 cells. A sensitive chemiluminescent assay showed that there was little β-galactosidase expression when enriched $CD34^+$ cells were transduced with Ad.CMV-βgal (~ 2,000 RLU/10^4 cells at a MOI = 10) and no β-galactosidase expression with Ad.DF3-βgal (Fig. 2 *B*). By contrast to the $CD34^+$ cells, bone marrow stromal cells were transduced efficiently by Ad.CMV-βgal (10^6 RLU/10^4 cells at a MOI = 10) (Fig. 2 *B*). Moreover, the stromal cells exhibited little β-galactosidase expression after transduction with Ad.DF3-βgal (Fig. 2 *B*). To substantiate the relatively low infectability of $CD34^+$ cells by adenovirus, we used PCR to determine the relative copy number of virus per cell after infection. Purified bright $CD34^+$ cells infected with adenovirus were obtained by fluorescence sorting and DNA was extracted for PCR analysis of adenoviral E2B sequences. There was no detectable adenovirus in 10^3 $CD34^+$ cells infected at a MOI of 10, while the E2B signal was readily apparent from 10^3 transduced MCF-7 cells (Fig. 2 *C*). A low level signal was obtained when assaying 10^4 infected $CD34^+$ cells (Fig. 2 *C*). By comparison with an adenovirus standard, we estimate that there are ~ 10 copies of virus per MCF-7 cell and < 0.01 copy per $CD34^+$ cell when cells were infected with adenovirus at a MOI of 10.

Adenovirus infection is a two-step process involving the initial attachment of adenoviral fiber protein to a relatively ubiquitously expressed, but yet unidentified, receptor and then internalization through interaction of the adenoviral penton base with αv integrins, particularly αvβ3 and αvβ5 heterodimers (35, 36). FACS® analysis indicated that $CD34^+$ cells had no detectable αv subunits, αvβ3, or αvβ5 (Table I). By contrast, αv subunits were strongly expressed on breast cancer, lung cancer, prostate cancer, and glioblastoma cells (Table I). The tumor cells expressed αvβ5 at high levels and αvβ3 to a lesser extent (Table I). These results indicated that the low level of adenoviral-mediated transduction in $CD34^+$, as compared to carcinoma, cells is attributable at least in part to the absence of integrins that contribute to adenoviral internalization.

Table I. FACS Analysis of Integrin αv Subunit and αvβ3, αvβ5 Heterodimers in $CD34^+$ Cells and Carcinoma Cells

Cell	Type	αv	αvβ3	αvβ5
$CD34^+$	Hematopoietic progenitor cells	−	−	−
MCF-7	Breast cancer	++++	−	+++
BT-20	Breast cancer	++++	−	++++
ZR-75	Breast cancer	++++	−	+++
SKBR3	Breast cancer	++++	−	+++
A549	Lung cancer	++++	+	+++
DU145	Prostate cancer	++++	++	+++
T98G	Brain glioblastoma	++++	++	+++

Cells were stained with mAbs for αv subunit, αvβ3, and αvβ5 heterodimers as described in Methods. Quantitation was determined as described (25) on the basis of fluorescence intensity. ++++, values above 60; +++, between 30 and 60; ++, between 10 and 30; +, values between 5 and 10; −, values under 5.

Table II. Adenovirus-mediated Reporter Gene Expression in Breast Cancer Cells Premixed with PB Mononuclear Cells

MCF-7 per 10^4 PB	RLU per 10^5 cells (mean±SEM) Ad.CMV-Luc	Ad.CMV-βgal	Ad.DF3-βgal
0	$1.1\pm0.2\times10^4$ (1)	$2.1\pm0.7\times10^3$ (1)	$0.5\pm0.3\times10^2$ (1)
2	$2.4\pm1.7\times10^4$ (2.3)	$3.5\pm0.4\times10^3$ (1.6)	$2.0\pm0.8\times10^2$ (4.0)*
10	$1.0\pm0.4\times10^5$ (10)*	$9.9\pm5.7\times10^3$ (5)*	$1.4\pm0.4\times10^3$ (28)*
10^2	$8.7\pm6.1\times10^5$ (83)‡	$4.9\pm3.4\times10^4$ (23)*	$4.4\pm0.8\times10^3$ (90)‡
10^3	$6.4\pm1.9\times10^4$ (615)‡	$1.4\pm0.5\times10^5$ (172)‡	$1.2\pm0.9\times10^5$ (2,359)‡
10^4	$3.2\pm1.7\times10^7$ (5,947)‡	$5.0\pm4.0\times10^6$ (2,354)‡	$0.6\pm0.3\times10^6$ (11,843)‡

MCF-7 cells were premixed with PB cells at the indicated ratios. The cells were incubated with Ad.CMV-Luc, Ad.CMV-βgal, and Ad.DF3-βgal at a MOI of 10 for 2 h at 37°C. At 48 h after infection, cells were harvested, lysed, and assayed for reporter activities using a luminometer. The reporter activities are presented as RLU per 100,000 cells (mean±SEM) obtained from four experiments. A background value of RLU from the uninfected cells was subtracted. The fold increase of reporter activity relative to tumor-free PB cells (MCF-7 = 0) is in parentheses (*$P \le 0.05$; ‡$P \le 0.001$).

The finding that adenovirus preferentially transduces carcinoma, as compared to hematopoietic, cells suggested that adenoviral-mediated reporter gene expression could be used to detect contaminating cancer cells in PB and BM. To address this issue, MCF-7 cells were premixed with PB cells at ratios of $1:10^2$ to $2:10^6$. Reporter activity of Ad.CMV-Luc- and Ad.CMV-βgal–infected cell mixtures reflected the number of contaminating MCF-7 cells (Table II). The level of luciferase activity mediated by Ad.CMV-Luc infection was significantly increased at a ratio of 10 MCF-7 cell/5 × 10^5 PB mononuclear cells. Higher ratios were associated with increases in reporter gene expression (Table II). Similar results were obtained with Ad.CMV-βgal (Table II). Studies performed with Ad.DF3-βgal demonstrated a lower background with uncontaminated PB mononuclear cells and enhanced sensitivity with detection of one MCF-7 cell/5 × 10^5 PB cells (Table II). Similar results were obtained with Ad.DF3-βgal when ZR-75-1 breast cancer cells were mixed with PB cells (data not shown). Other studies were performed on BM cells that had been contaminated (0.1%) with DF3/MUC1-positive breast, lung, prostate, and ovarian cancer cells. The contaminated BM cells demonstrated a marked elevation in reporter activity when using Ad.DF3-βgal (Fig. 3 *A*). Furthermore, contamination of BM with increasing numbers of MCF-7 cells resulted in higher levels of Ad.DF3-βgal–mediated reporter gene expression, while there was no increase in β-galactosidase expression when the BM cells were contaminated with DF3/MUC1 negative T98G glioblastoma cells (Fig. 3 *B*).

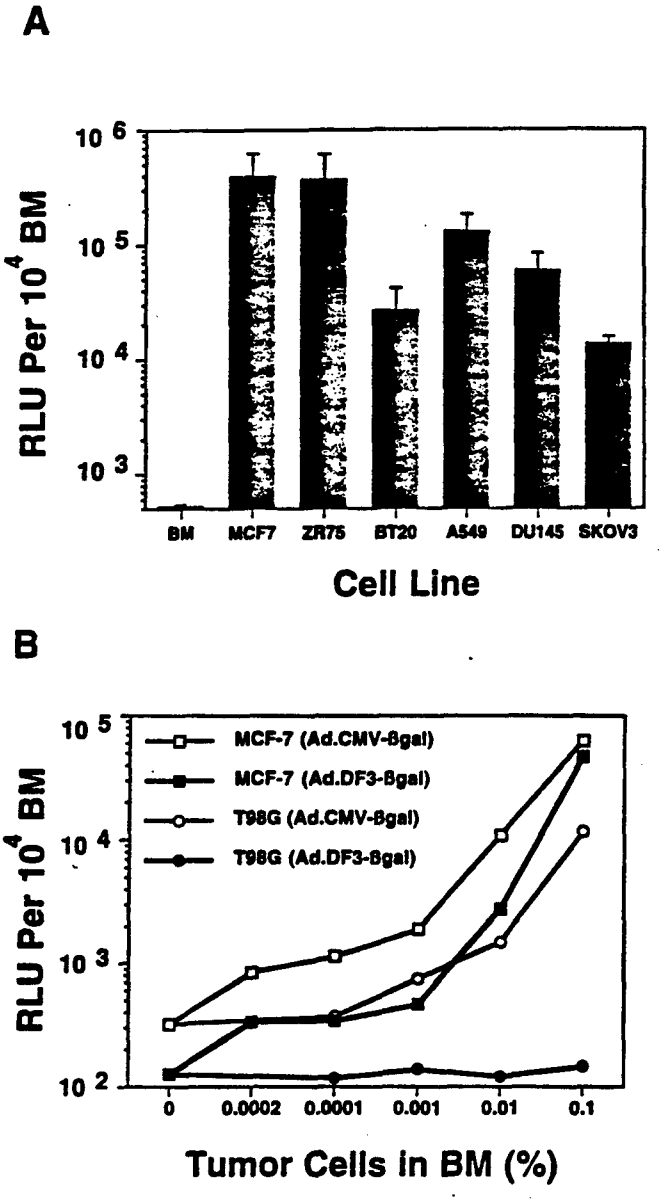


Figure 3. Adenovirus-mediated reporter gene expression in BM contaminated with cancer cells. (*A*) BM mononuclear cells were mixed with 0.1% MCF-7, ZR-75, BT-20 (*breast cancer*), A549 (*lung cancer*), DU145 (*prostate cancer*), and SKOV3 (*ovarian cancer*) cells. The cells were incubated with Ad.DF3-βgal at a MOI of 10 for 2 h at 37°C. After 24 h, cells were lysed and assayed for reporter gene expression by chemiluminescence assay. (*B*) BM mononuclear cells premixed with various ratios of MCF-7 or T98G cells were incubated with either Ad.CMV-βgal or Ad.DF3-βgal at a MOI of 1 for 2 h at 37°C, washed, and cultured for 24 h. β-galactosidase expression was measured by chemiluminescence assay. Similar results were obtained in three separate experiments.

To extend the observation of selective adenoviral-mediated reporter gene expression, we explored other approaches for detection of contaminating carcinoma cells. BM mononuclear cells with and without contaminating MCF-7 cells were infected with Ad.DF3-βgal and then visualized for X-gal staining. Using this approach, the MCF-7 cells could be readily identified by blue staining (Fig. 4, *A* and *B*). The contaminating cells were also readily apparent by fluorescence microscopy after staining with the fluorescence substrate FDG (Fig. 4, *C* and *D*). Cells that expressed β-galactosidase also reacted with mAb DF3 (data not shown), a monoclonal antibody that detects DF3/MUC1 (21). These findings indicated that histochemical, as well as biochemical, approaches can be used for detection of contaminating tumor cells by adenoviral-mediated reporter gene expression.

The selectivity of adenoviral-mediated gene transduction for contaminating tumor cells supported the possibility of using this approach to purge hematopoietic cell populations. Previous studies have documented the strategy of expressing the *HSV-tk* gene for selective killing by GCV (20). To exploit this strategy for purging, adenovirus carrying HSV-tk under control of the CMV or DF3/MUC1 promoters was used to transduce PB cells premixed with tumor cells. As determined by clonogenic survival, infection at an MOI of 10 followed by GCV treatment (10 to 1,000 μM) resulted in the elimination of over 6 logs of contaminating MCF-7 cells. Infection with Ad.DF3-tk at a MOI of 100 and then treatment with 100 μM GCV killed ~6 logs of cancer cells (Fig. 5 *A*). In addition, this approach effectively eliminated other contaminating breast, prostate, lung, and glioblastoma tumor cells premixed with BM cells (Fig. 5 *B*).

A potential adverse effect of ex vivo purging is toxicity to hematopoietic progenitor cells. We thus assessed the effects of adenoviral infection and GCV treatment on CFU-GM and BFU-E. Infection with Ad.CMV-tk or Ad.DF3-tk at a MOI of 10 followed by GCV (100 μM) treatment had little effect on CFU-GM and BFU-E as compared with GCV alone (≤ 10% cytotoxicity). Adenovirus infection alone at a MOI of 10 had little if any effect on colony formation (Table III). At a MOI of 100, there was a 17–19% decrease in BFU-E and CFU-GM when Ad.CMV-tk and GCV were used, while there was less of an effect with Ad.DF3-tk and GCV (Table III). Limiting dilution assays were also performed on enriched CD34⁺ cells to assess the effects of adenovirus and GCV treatment on LTC-ICs. The results demonstrate that infection with Ad.CMV-tk with or without GCV treatment has little if any effect on the regeneration and differentiation of the primitive progenitor cells

Table III. Progenitor Cell Growth of CD34⁺ Cells Treated with Adenovirus and GCV

Treatment	BFU-E	CFU-GM
Untreated	100	100
+GCV	91±2.8*	90±2.5*
+Ad.DF3-tk (MOI=10)	102±4.1	100±3.2
+Ad.DF3-tk (MOI=10) + GCV	91±3.9	91±2.8*
+Ad.DF3-tk (MOI=100) + GCV	90±3.3*	90±3.0*
+Ad.CMV-tk (MOI=10)	100±3.2	100±3.1
+Ad.CMV-tk (MOI=10) + GCV	90±3.0*	89±3.0*
+Ad.CMV-tk (MOI=100) + GCV	83±3.9‡	81±4.0‡§

Enriched CD34⁺ cells were treated with the indicated adenovirus for 24 h at 37°C, washed, and cultured for 24 h. GCV (100 μM) was then added for 24 h. The cells were washed and then cultured in methylcellulose for 2 wk. The number of colonies in the treated groups is expressed as the percentage (mean±SEM from four experiments) of that for untreated controls (21.8±7.3 BFU-E and 26±5.9 CFU-GM per 1,000 CD34⁺ cells). *$P \leq 0.05$, ‡$P \leq 0.001$ vs untreated control; §$P \leq 0.05$ vs GCV alone.

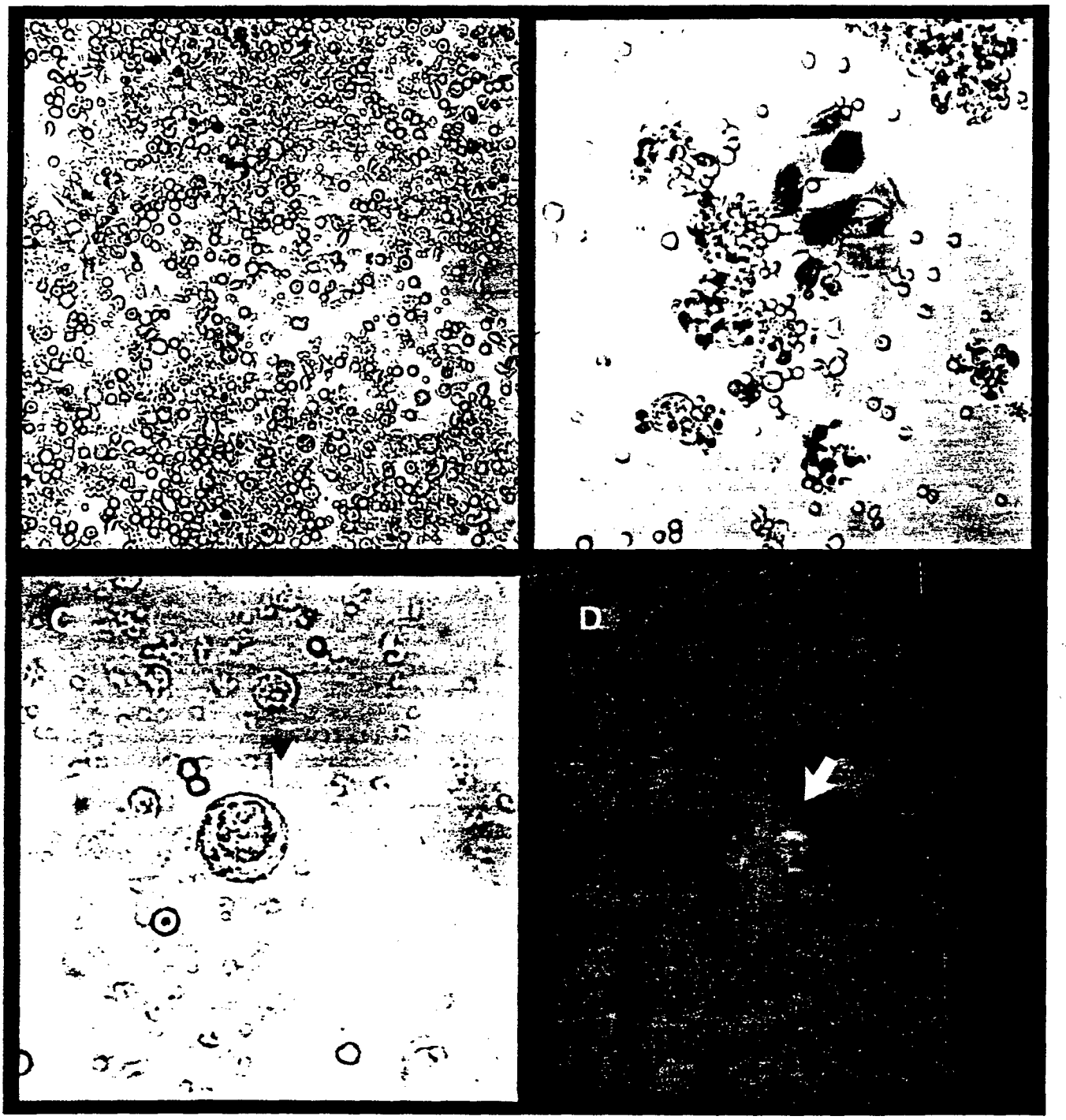


Figure 4. Detection of contaminating breast cancer cells in BM by Ad.DF3-βgal. Cells were incubated with Ad.DF3-βgal (MOI = 10) for 2 h at 37°C, washed, cultured for 24 h, fixed and then stained with X-gal. (*A*) BM mononuclear cells without MCF-7 cells. (*B*) BM mononuclear cells containing 0.1% MCF-7 cells. Magnification is 400. Cells were also incubated with FDG and observed under a fluorescent microscope. (*C*) Bright field. (*D*) Dark field. Magnification is 1000. Arrows indicate breast cancer cells.

(Fig. 6). Additional experiments were performed to determine if adenovirus is detectable in the progeny cells after adenoviral purging of progenitor cells. CFU-GMs and BFU-Es were picked from methycellulose and cultured with 293 cells. No live adenovirus was rescued in three separate experiments. Reverse transcription PCR analysis of CFU-GM and BFU-E colonies failed to detect any transgene expression mediated by recombinant adenovirus (data not shown). Importantly, the finding that PCR analysis did not detect the presence of adenoviral E1a sequences indicated no wild-type adenovirus replication.

Discussion

A major issue for autologous BM or PB transplantation in breast cancer patients is the potential risk of collecting and re-

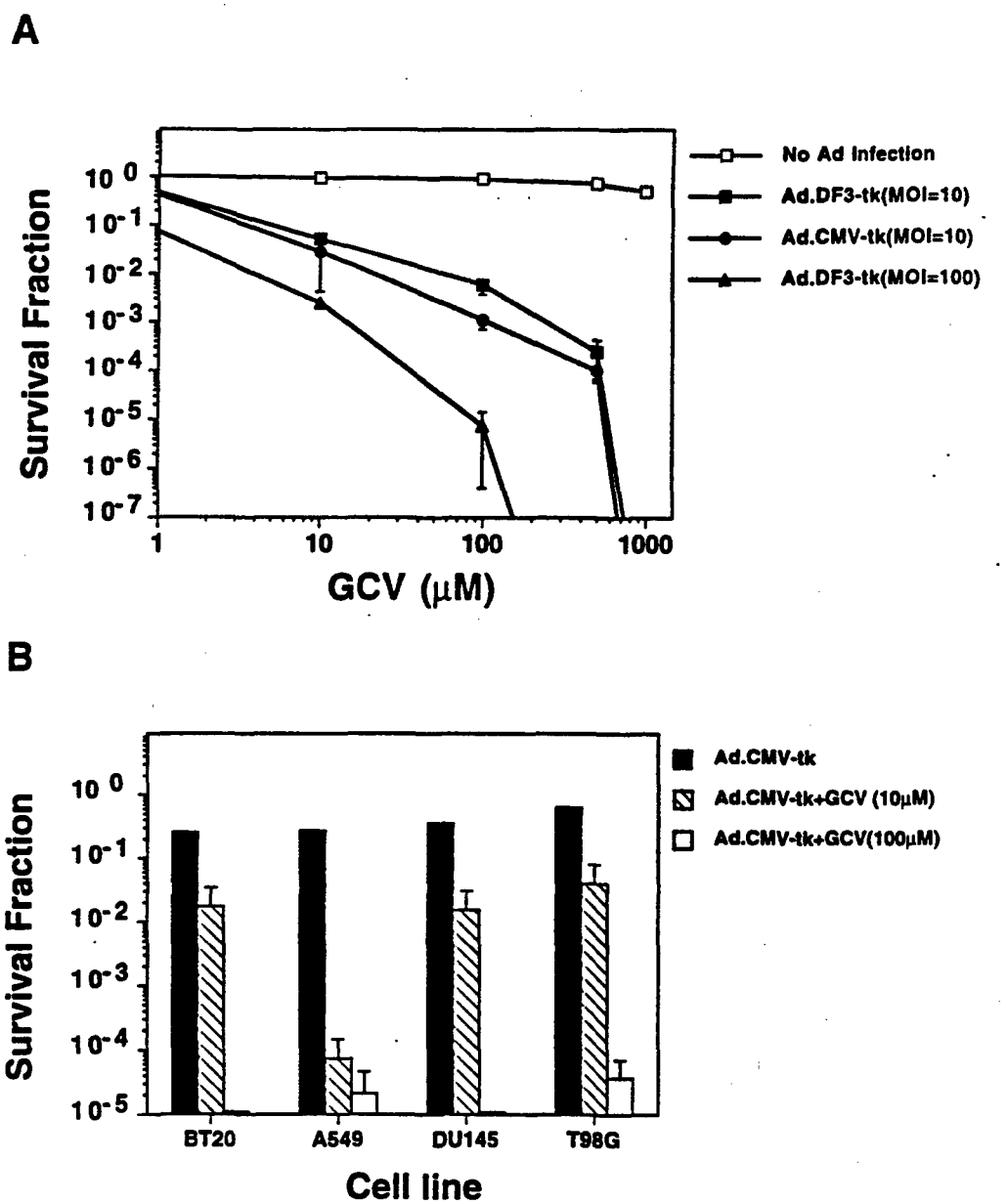


Figure 5. Effects of Ad.CMV-tk and Ad.DF3-tk infection followed by GCV treatment on the survival of cancer cells premixed in PB or BM mononuclear cells. Cancer cells were premixed with 10-fold excess of irradiated PB or BM cells. The cells were then incubated with recombinant adenoviruses at 37°C for 2 h. At 24 h after infection, cells were treated with GCV at the indicated concentrations for 24 h, and were then replated on 30-mm plates in duplicate at serial dilutions ranging from 500 to 10^4 cells per well. 2 wk later, the number of colonies (> 50 cells) was assessed by crystal violet staining. (*A*) Clonogenic assay for MCF-7 breast cancer cells premixed in PB and infected with Ad.CMV-tk or Ad.DF3-tk at the indicated MOIs followed by GCV treatment. The results are expressed as survival fraction, i.e., colony numbers in plates treated with adenovirus and/or GCV as a fraction of that for untreated controls (mean±SEM for two to four experiments). (*B*) Clonogenic assay for carcinoma cells premixed in BM treated with Ad.CMV-tk at a MOI of 10 and GCV.

infusing tumor cells. In this context, a study using histochemical detection has demonstrated BM involvement in 50% of patients with localized breast cancers and both BM (70%) and PB (22.5%) involvement in patients with stage IV disease (5). Gene transfer may provide one strategy for improving the detection and purging of tumor cells in BM or PB preparations. However, the presently available gene delivery systems generally lack target cell specificity. Ligand-DNA complexes, DNA-liposome complexes, and direct transfer of DNA are limited by a low efficiency of gene transduction (37–40). Moreover, the use of retroviral vectors for detection or purging of cancer cells in hematopoietic stem cell preparations could be limited by dependence on replication of the target cell. By contrast, replication-defective adenoviral vectors represent a highly efficient approach for in vitro gene transfer. One potential limitation of this vector system could be transduction of reporter or therapeutic genes into hematopoietic as well as tumor cells. However, the present studies demonstrate that adenovirus is markedly inefficient in the transduction of BM and PB, as compared with carcinoma, cells. Importantly, transduction of purified $CD34^+$ hematopoietic stem and progenitor cells is also inefficient compared with that of cancer cells. Another study has recently reported similar results in BM and $CD34^+$ cell preparations (41). Our results further indicate that the $CD34^+$ cell populations express low to undetectable levels of the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins. Internalization of adenovirus requires interaction of the adenoviral penton base with αv integrins (35, 36). Consequently, the absence of detectable αv integrin subunits on $CD34^+$ cells and their high level expression on diverse cancer cells provides a mechanistic explanation for the selectivity of transduction.

The finding that adenoviral-mediated gene transduction is inefficient in BM and PB cell preparations compared to carcinoma cells supported the potential for using this approach to detect contaminating tumor cells. Transduction of the luciferase or β-galactosidase genes demonstrated a correlation

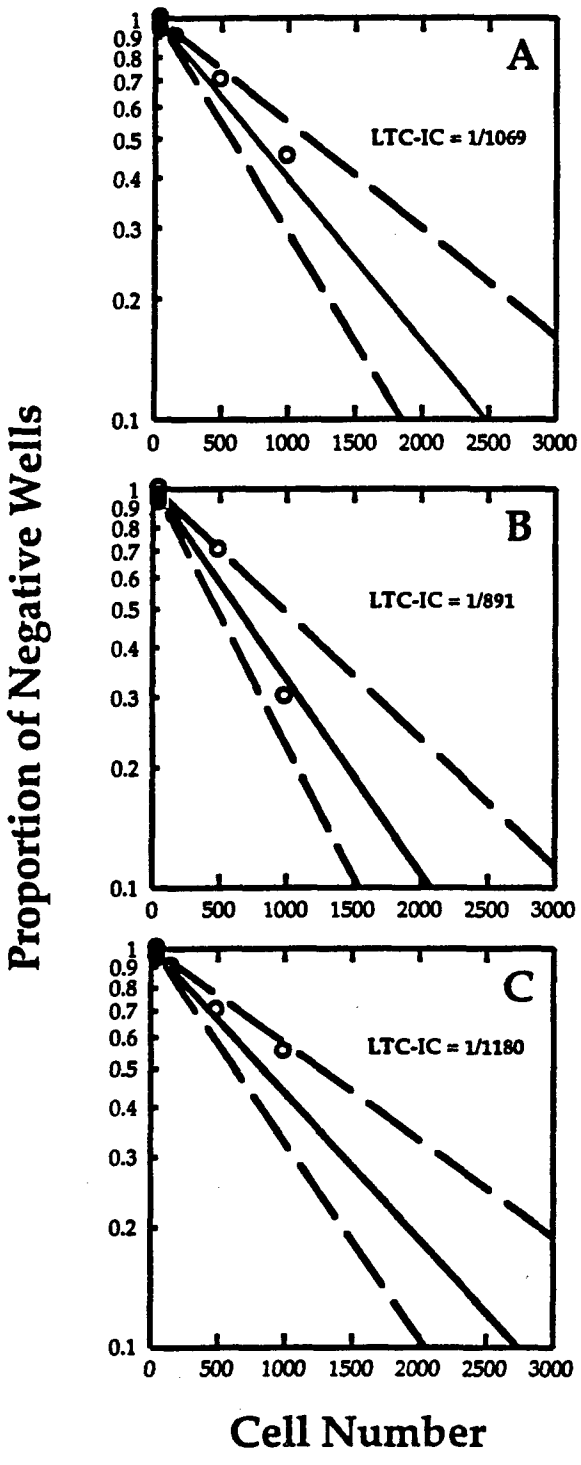


Figure 6. Limiting dilution analysis of LTC-ICs for primitive progenitor cells. Enriched CD34$^+$ cells were treated as described in Table III, and seeded onto irradiated marrow feeders in 96-well plates at concentrations of 55 to 4,000 cells/well and 20 wells per concentration. The number of clonogenic wells was assessed after 5 wk of suspension culture and 2 wk of growth in methylcellulose. The frequency of LTC-ICs was calculated by plotting the input cell number against the proportion of negative wells. The best linear fit and standard errors were determined from the data. (*A*) Untreated control. (*B*) Ad.CMV-tk infection (MOI = 10). (*C*) Ad.CMV-tk (MOI = 10) and GCV (100 μM) treatment. Similar results were obtained in two independent experiments.

between reporter expression and the number of contaminating cells. Expression of the reporter resulted in the detection of one cancer cell in 5×10^5 BM or PB cells. By contrast, an immunohistochemical method using antibodies against cytokeratin detects one tumor cell in 4×10^5 BM cells (5). Moreover, we found that Ad.DF3-βgal transduction can be adapted for histochemical detection and thereby morphological examination by staining with X-gal or FDG. Furthermore, the use of the tumor-selective promoter resulted in lower backgrounds with uncontaminated hematopoietic cell preparations. We previously demonstrated that use of the DF3 promoter in adenoviral vectors provides an efficient and selective approach to target expression of heterologous genes in breast cancer cells (20). There are presently several other tumor-specific or selective promoter sequences that have been used to confer selective expression of heterologous genes in tumor cells (42–45). The present results suggest that use of a tumor-selective promoter in the context of an adenoviral vector can provide a highly sensitive approach for the detection of cancer cells in hematopoietic cell preparations. Studies will now be needed that directly compare the sensitivity of the present approach with other techniques used for detection of contaminating carcinoma cells.

The differential sensitivity of hematopoietic as compared to carcinoma cell transduction by adenoviral vectors further supported the use of this approach to purge contaminating tumor cells. Previous studies have demonstrated that purging BM preparations with 4-hydroperoxycyclophosphamide (4-HC) can lead to 2–3 logs of tumor cell depletion (12, 17). The use of immunomagnetic separation in combination with 4-HC eliminated up to 5 logs of tumor cells (12). However, this approach significantly reduced the recovery of CFU-GM (12). mAbs linked with toxin proteins have also been used for in vitro purging of bone marrow. mAb DF3 linked to ricin resulted in the elimination of 2–3 logs of breast tumor cells (13). However, this approach also resulted in the reduction of CFU-GM formation. The present studies demonstrate that adenoviral mediated gene transduction using Ad.DF3-tk and GCV treatment results in the elimination of 6 logs of contaminating breast cancer cells. Importantly, there was little effect of Ad.DF3-tk transduction and GCV treatment on recovery of CFU-GM and BFU-E. Moreover, the adenovirus-mediated transduction of thymidine kinase to confer GCV sensitivity had little effect on LTC-ICs of enriched CD34$^+$ cells. Since completion of the present studies, another report has demonstrated that adenoviral vectors expressing wild type p53 can be used to purge breast cancer cells mixed with normal bone marrow (41). Other studies have demonstrated that adenoviruses can be used to selectively transduce cancer cells with genes that induce apoptosis (46) and to increase transduction of plasmid vectors coding for toxin genes (41). Thus, combining several adenoviral-mediated strategies could be useful in increasing the efficacy of purging contaminating cancer cells in hematopoietic cell preparations.

Finally, the present results suggest that adenoviral-mediated gene transduction could be useful for the detection and elimination of diverse carcinomas contaminating bone marrow and peripheral blood collections. In addition to studies with breast cancer cells, adenoviral-mediated transduction was highly efficient for cells derived from lung, prostate, and ovarian carcinomas. As the DF3/MUC1 antigen is overexpressed in breast, lung, prostate, and ovarian cancers (21–23), adenovi-

ral vectors containing the DF3/MUC1 promoter could be used in these settings to further increase selectivity of gene transduction. Alternatively, other tumor-selective DNA regulatory elements can be used in a similar context. The present results support the use of replication defective adenoviral vectors with the DF3/MUC1 promoter for purging hematopoietic cell preparations in the clinical setting.

Acknowledgments

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Editorial

A concept fundamental to human gene therapy is efficient and specific somatic gene transfer. A generous assessment of the field would conclude that most vectors fall short of the mark, although a few illustrative exceptions indicate some progress has been made. Recombinant adenoviruses have provided the most encouraging results as of late (1). Vectors based on human adenoviruses are extraordinarily efficient gene transfer vehicles in a wide variety of cells both in vitro and in vivo. A glaring exception is hematopoietic derived cells which seem virtually impenetrable to these vectors (2). Chen et al. describe in this issue of *The Journal* a potential application of adenoviruses to purge bone marrow of tumor cells that is based on the relative resistance of bone marrow progenitors to adenoviral vectors (3).

Autologous bone marrow transplantation is having an expanding role in the treatment of malignancies such as breast cancer. Patients are treated with high dose chemotherapy and the resulting pancytopenias are corrected by transplanting autologous bone marrow cells harvested before the therapy. Patients receiving autologous bone marrow tolerate doses of chemotherapy that otherwise would be lethal. Remission has been achieved in some patients although virtually all eventually relapse. A potential source of relapse is outgrowth of tumor cells that contaminate the transplanted bone marrow cells. This problem has led to strategies to selectively purge the bone marrow of tumor cells before transplantation; most approaches suffer from insufficient specificity resulting in incomplete purging of cancer cells and clinical relapses, or toxicity to the bone marrow and incomplete hematopoietic reconstitution.

The study by Chen et al. describes a novel approach for purging breast cancer cells from bone marrow that is based on somatic gene transfer (3). They exploited the apparent failure of adenoviruses to infect hematopoietic cells to selectively target the more infectable breast cancer cells with a "suicide gene." Human bone marrow contaminated with variable quantities of breast cancer cells was exposed to an adenoviral vector expressing the thymidine kinase (TK) gene from Herpes Simplex Virus. Selective ablation of vector transduced cells was achieved in the presence of ganciclovir which, in cells expressing TK, is converted to a toxic phosphorylated metabolite (4). The specificity by which this was achieved was impressive, infecting ~ 1 cancer cell in 5×10^5 bone marrow cells. This was accomplished without compromising the viability of bone marrow progenitors.

The application of adenoviral vectors for bone marrow purging in humans should consider several issues. The adenoviral capsid proteins, per se, have demonstrated toxicity to a variety of cells independent of transduction. It will be necessary to assure that the ability of stem cells to fully reconstitute in all lineages is not affected by the ex vivo infection protocol. Furthermore, it remains to be seen if the purging efficiency is sufficient to eliminate the contaminating tumor cells. In vitro studies have demonstrated significant variation in the relative infectability of different tumor isolates, suggesting there may be heterogeneity in clinical responses.

The study by Chen et al. is important to the field of gene therapy for several reasons. Despite the caveats noted above, this application of gene transfer technology has real therapeutic potential. The problem of relapse following autologous bone marrow transplantation in cancer is a substantial clinical problem with no obvious solutions, thereby justifying novel approaches. All manipulations occur ex vivo so that immunologic responses to the vector and vector gene products, a problem that has plagued in vivo approaches (5), are irrelevant. The actual weakness of the vector (i.e., poor gene transfer in hematopoietic cells) is exploited to improve specificity. Finally, there is a growing experience in humans confirming the safety of adenoviral vectors (6).

This use of adenoviral vectors in this application is a poignant example how far the field of gene therapy has come since the 1980s when it was solely considered in the context of gene replacement for the treatment of autosomal recessive diseases. A full spectrum of more common acquired diseases has been considered for gene therapy. Substantial effort has been directed to the use of gene transfer in the treatment of malignancy with some of the most promising strategies attempting to enhance anticancer immunity through vaccines or adoptive transfer. Similarly creative programs have been developed for the genetic treatment of cardiovascular diseases, AIDS, and auto-immune diseases.

Gene transfer vectors have emerged as powerful tools to study and potentially treat diseases. The concept is fundamental and the technology is evolving in step with the spectacular evolution of biomedical research. The challenge is to identify those clinical situations in which value is gained by incorporating the transfer of genetic material. The study by Chen et al. is an elegantly simple application. Only time will tell if it will impact on the outcome of autologous bone marrow transplantation for cancer.

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Induction of antigen-specific antitumor immunity with adenovirus-transduced dendritic cells

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Transduction of dendritic cells (DC) can result in presentation of tumor-associated antigens and induction of immunity against undefined epitopes. The present studies demonstrate adenovirus (Ad)-mediated transduction of the β-galactosidase gene in mouse DC. Similar transductions have been obtained with the gene encoding the DF3/MUC1 tumor-associated antigen. We show that the Ad-transduced DC are functional in primary allogeneic mixed lymphocyte reactions. Mice immunized with Ad-transduced DC develop cytotoxic T lymphocytes that are specific for the β-galactosidase or DF3/MUC1 antigens. The results also demonstrate that Ad.MUC1-transduced DC induce a specific response which inhibits the growth of DF3/MUC1- positive tumors. These findings support the usefulness of Ad-transduced DC for in vivo immunization against tumor-associated antigens.

Keywords: *dendritic cells; adenovirus; transduction; antitumor immunity; DF3/MUC1*

Introduction

Dendritic cells (DC) are potent antigen-presenting cells (APCs) that have the capacity to activate naive cytotoxic T cells.[1] Murine DC pulsed with peptides prime antigen-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) *in vivo*.[2] Peptides derived from tumor-associated antigens have similarly been used to pulse DC and induce antitumor immunity.[3–5] Other studies have employed soluble tumor-associated antigens for loading DC and generating antitumor activity.[6] Whereas peptides pulsed on to DC may dissociate from MHC molecules, $CD34^+$ cells have been retrovirally transduced to stably express antigens after differentiation to DC.[7,8] In contrast to pulsing, transduction of DC can result in longer term antigen presentation and induction of immunity against undefined MHC epitopes. Thus, transduced DC may be effective in immunizing against known tumor-associated antigens.

The human DF3/MUC1 glycoprotein is aberrantly overexpressed in breast and other carcinomas.[9] The DF3 protein is one member of the MUC1 family of carcinoma-associated antigens that contain variable numbers of highly conserved (G+C)-rich 60 base pair tandem repeats.[10,11] A C-terminal region includes a transmembrane domain that anchors the antigen at the cell surface.[12,13] Cell–cell interactions are reduced in cells transfected with the MUC1 cDNA.[14] Other work has demonstrated that DF3 inhibits the recognition of targets by immune effector cells.[15–17] These findings have suggested that the DF3/MUC1 tumor-associated antigen may function in inhibiting antitumor immunity.

The present studies demonstrate adenovirus-mediated transduction of the β-galactosidase and DF3/MUC1 genes in mouse DC. We also show that the transduced DC are functional in inducing antitumor immunity.

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Results and discussion

Flow cytometry was used to define the phenotype of DC following transduction with recombinant adenovirus. DC derived from bone marrow expressed MHC class I and II products, costimulatory molecules and ICAM-1[18] (Figure 1a). Transduction with Ad.βgal resulted in a similar pattern of antigen expression (Figure 1a). Moreover, transduction with Ad.MUC1 was associated with DF3/MUC1 expression and little if any effect on cell surface levels of MHC, costimulatory or adhesion molecules (Figure 1a). The Ad.MUC1-transduced DC exhibited a typical morphology with veiled dendrites (Figure 1b). Staining with MAb M5/114 (anti-MHC class II) and MAb DF3 demonstrated expression of DF3/MUC1 by the transduced DC (Figure 1b). Immunoblot analysis of the Ad.MUC1 transduced DC confirmed DF3/MUC1 expression (Figure 1c). Whereas MAb DF3 detects glycosylated MUC1, the finding that MAb DF3-P reacts with an approximately 55 kDa protein in the transduced DC also provides support for detection of the unglycosylated protein core[19] (Figure 1c).

DC are potent stimulators of primary mixed lymphocyte reactions (MLR).[20,21] To assess in part the function of Ad-transduced DC, we compared their effects in primary allogeneic MLR with those obtained from non-transduced DC. The results demonstrate that DC transduced with Ad.MUC1 or Ad.βgal at MOI of 100 exhibit the same potent stimulatory function as control DC (Figure 2a). By contrast, DC transduced at MOIs of 200 or 500 exhibited decreases in viability (data not shown) and in T cell stimulation (Figure 2b). These results indicate that expression of adenoviral, rather than the transgene, proteins is responsible for the loss of DC function.

To determine whether Ad-transduced DC induce antitumor immunity, we immunized mice twice with un-

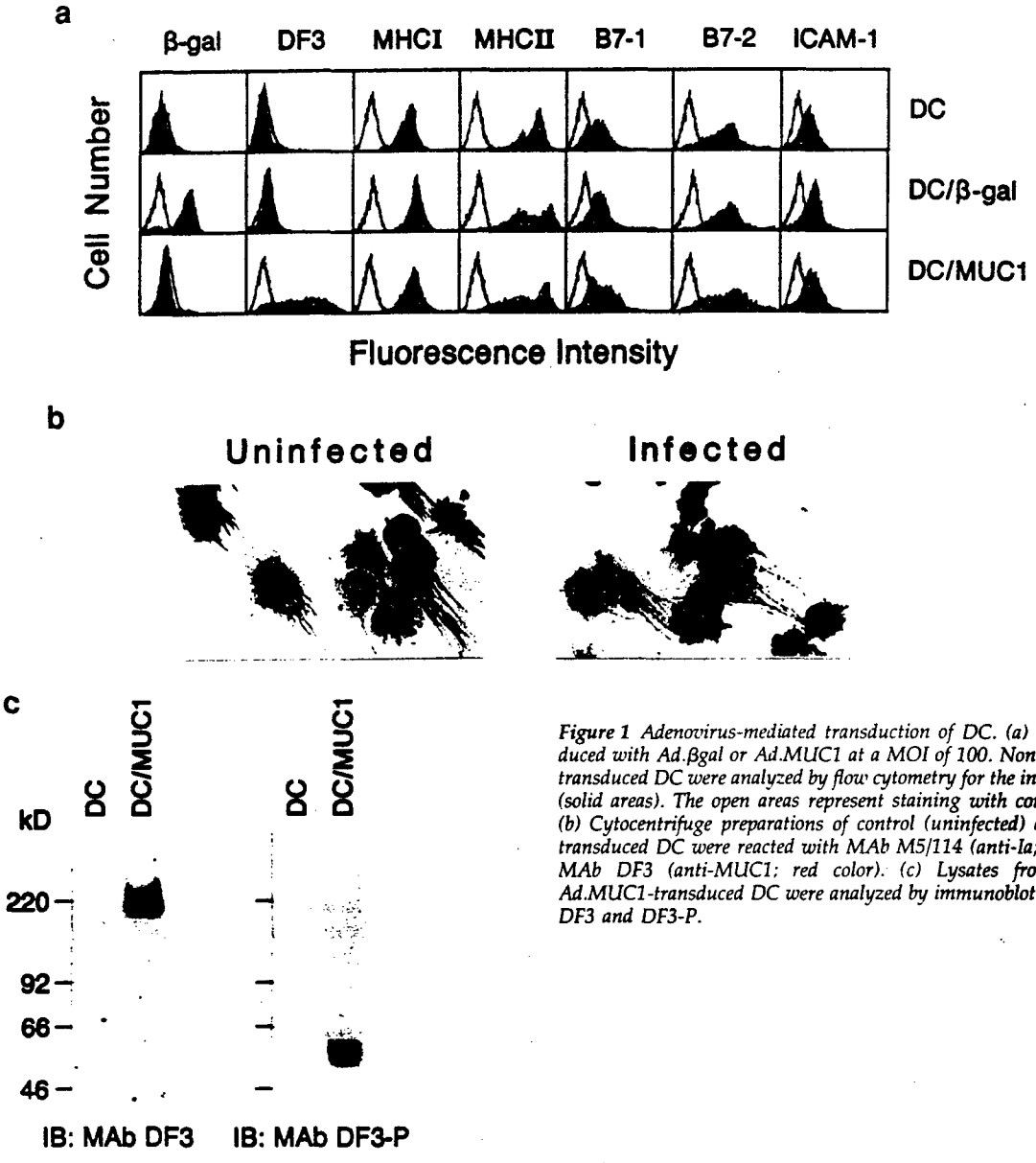

Figure 1 Adenovirus-mediated transduction of DC. (a) DC were transduced with Ad.βgal or Ad.MUC1 at a MOI of 100. Non-transduced and transduced DC were analyzed by flow cytometry for the indicated antigens (solid areas). The open areas represent staining with control antibodies. (b) Cytocentrifuge preparations of control (uninfected) and Ad.MUC1-transduced DC were reacted with MAb M5/114 (anti-Ia; blue color) and MAb DF3 (anti-MUC1; red color). (c) Lysates from control and Ad.MUC1-transduced DC were analyzed by immunoblotting with MAbs DF3 and DF3-P.

infected DC, Ad.MUC1-transduced DC or Ad.βgal-transduced DC. Splenocytes were assayed for CTL activity using as targets syngeneic MC-38 carcinoma cells that stably express DF3/MUC1.[22] T cells from mice immunized with Ad.MUC1-transduced DC exhibited strong activity against MC-38/MUC1, but not wild-type MC-38, cells (Figure 3a). CTLs from these mice also induced lysis of Ad.MUC1-, and not Ad.βgal-transduced, MC-38 cells (Figure 3a). By contrast, T cells from mice immunized with Ad.βgal-transduced DC exhibited lysis of only the Ad.βgal-transduced MC-38 cells (Figure 3b). These findings indicated that Ad-transduced DC induce immunity which is directed against the transgene.

Incubation of CTLs from mice immunized with Ad.MUC1-transduced DC with anti-CD4 or anti-CD8 antibodies blocked lysis of the MC-38/MUC1 targets (Figure 4a). These results indicated that Ad.MUC1-transduced DC generate MHC class I and II-restricted T cell responses. The finding that incubation of MC-38/MUC1 targets with MAb DF3 blocks lysis provided further support for specificity against DF3/MUC1 (Figure 4a). Moreover, incubation of YAC-1 cells with the CTLs showed no specific lysis (data not shown). Immunization of mice with Ad.MUC1-transduced DC also inhibited growth of MC-38/MUC1 tumors, while Ad.βgal-transduced or non-transduced DC had no effect on tumor growth (Figure 4b). To assess the potency of the Ad-transduced DC, we compared induction of immunity with that obtained when using monocytes. The efficiency of Ad.βgal- and Ad.MUC1-mediated transduction of DC and monocytes was similar (data not shown). However, immunization with Ad.MUC1-transduced monocytes was less effective than Ad.MUC1-transduced DC in the induction of CTL activity (Figure 4c). Ad.MUC1-transduced monocytes were also less effective than the transduced DC in inhibiting the growth of MC38/MUC1 tumors (Figure 4d). These findings are in concert with the greater potency of DC as APCs.[1]

Previous studies have demonstrated retroviral transduction of human $CD34^+$ progenitor cells and then

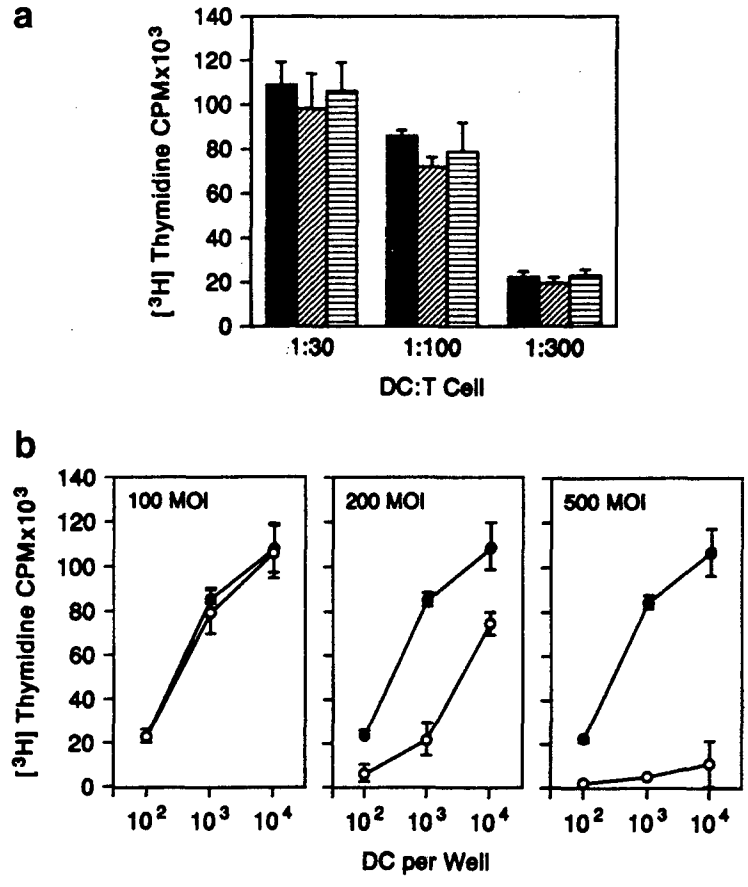

Figure 2 Induction of T cell proliferation responses with adenovirus-transduced DC. (a) Non-transduced DC (solid bars) and DC transduced (MOI = 100) with Ad.βgal (diagonal bars) or Ad.MUC1 (horizontal bars) were irradiated and then incubated at the indicated ratios with 2×10^5 allogeneic Balb/c T cells. The cells were cocultured in the MLR for 5 days. ^{3}H-thymidine uptake was assessed for 6 h at the end of coculturing. The results are expressed as the mean ± s.e. of three experiments each performed in triplicate. (b) Non-transduced DC (●) and DC transduced with Ad.MUC1 at MOIs of 100, 200 and 500 (○) were incubated with 1×10^5 Balb/c T cells. The results are expressed as the mean ± s.e. of three experiments each performed in triplicate.

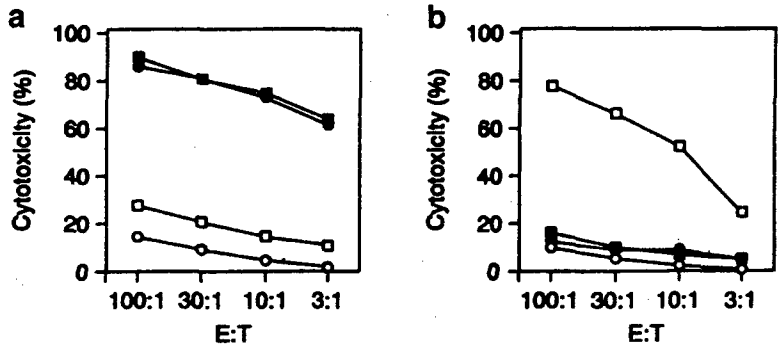

Figure 3 CTL activity of mice immunized with adenovirus-transduced DC. C57Bl/6 mice were immunized with 5×10^5 Ad.MUC1- (a) or Ad.βgal-transduced (b) DC on days 1 and 10. Splenocytes isolated on day 20 were incubated with MC-38 (○), MC-38/MUC1 (●), Ad.MUC1-transduced MC-38 (■) or Ad-βgal-transduced MC-38 (□) cells at the indicated ratios. CTL activity was determined by the 4 h LDH release assay. The results are expressed as percentage cytotoxicity determined from three experiments each performed in triplicate.

differentiation of the transduced cells into DC by cytokine stimulation.[7,8] The differentiated DC expressed the transgene and were functional in stimulating T cells *in vitro*.[7,8] Whereas retroviral transduction requires proliferating cells, adenovirus-transduced gene expression is not dependent on cell growth. The present studies demonstrate that murine DC can be efficiently transduced by adenoviral vectors. Transduction of DC with Ad.MUC1 or Ad.βgal at a MOI of 100 resulted in over 80% of the cells expressing the transgene. Similar transduction efficiencies were obtained in monocytes and fibroblasts. Whereas transduction at a MOI of 100 had no effect on stimulation in the MLR assay, higher MOIs (200 and 500) resulted in lower levels of T cell proliferation. This finding was associated with cytopathic effects observed at the higher MOIs.

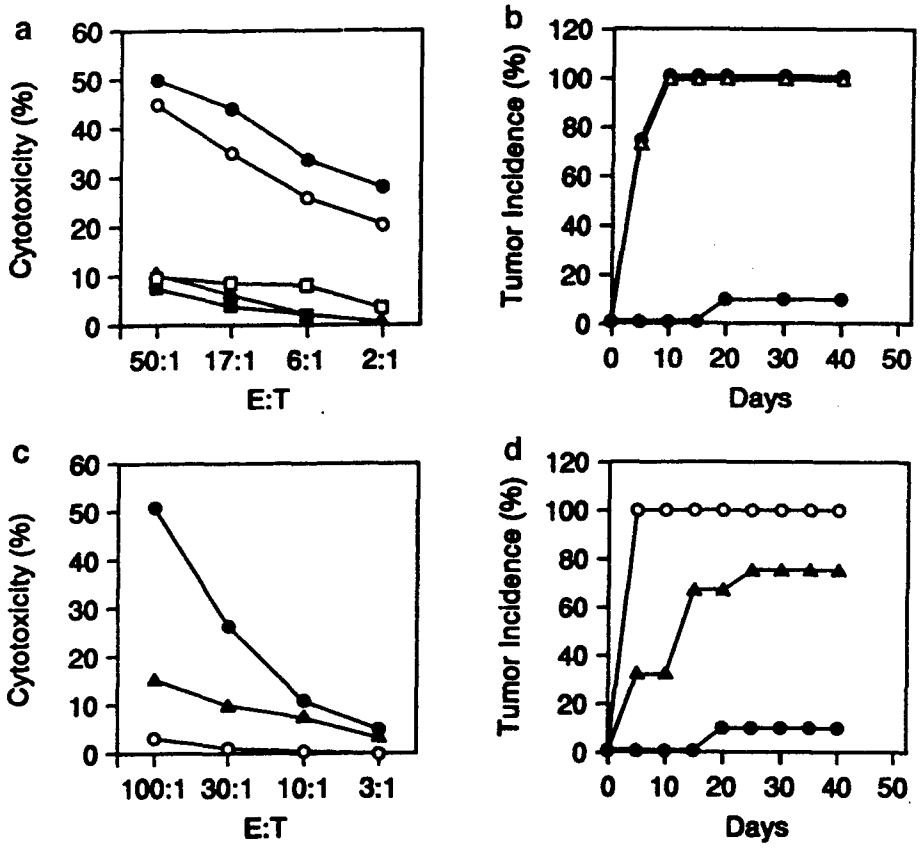

Figure 4 Antitumor activity of mice immunized with adenovirus-transduced DC or monocytes. (a) Mice were immunized twice with 5×10^5 Ad.MUC1-transduced DC. Splenocytes isolated on day 20 were incubated with no antibody (●), rat IgG (○), anti-CD4 (▲) or anti-CD8 (■) for 1 h at 4°C. Splenocytes were incubated with MC-38/MUC1 cells at the indicated ratios. MC-38/MUC1 cells were also incubated with 25 μg/ml MAb DF3 and then mixed with splenocytes otherwise not exposed to antibody (□). CTL activity was determined by the 4 h LDH release assay for three experiments each performed in triplicate. (b) Groups of 10 mice were immunized twice with 5×10^5 DC (△), Ad.MUC1-transduced DC (●) or Ad.βgal-transduced DC (○). The mice were then challenged with subcutaneous injections of 2×10^5 MC-38/MUC1 tumor cells. Tumor growth >3 mm in diameter was scored as positive. Similar results were obtained in four separate experiments. (c) Mice were immunized twice with 5×10^5 DC (○), Ad.MUC1-transduced DC (●) or Ad.MUC1-transduced monocytes (▲). Splenocytes were incubated with MC-38/MUC1 cells at the indicated ratios. CTL activity was determined by the 4-h LDH release assay for three experiments each performed in triplicate. (d) Groups of 10 mice were immunized twice with 5×10^5 DC (○), Ad.MUC1-transduced DC (●) or Ad.MUC1-transduced monocytes (▲). The mice were then challenged with subcutaneous injections of 2×10^5 MC-38/MUC1 cells. Tumor growth >3 mm in diameter was scored as positive.

Studies with retrovirally transduced $CD34^+$ cells that differentiate to DC have not been performed in an animal model; therefore, it is not known whether these cells are useful for *in vivo* immunization. The present studies demonstrate that immunization with Ad.MUC1- or Ad.βgal-transduced mouse DC induce CTL responses that are specific for the transgene. Reactivity against adenoviral antigens was apparently low based on the selectivity of the CTL response against DF3/MUC1 or βgal. Treatment of the CTLs with antibodies against T cell subsets indicated that the Ad-transduced DC stimulate a $CD4^+$ and $CD8^+$ immune response. Significantly, induction of anti-DF3/MUC1 immunity with the Ad.MUC1-transduced DC was sufficient specifically to inhibit the growth of DF3/MUC1-positive tumor cells. These findings support the usefulness of Ad-transduced DC for *in vivo* immunization against tumor-associated antigens.

Materials and methods

Cell culture

DC were isolated from bone marrow cultures as described.[18] Briefly, bone marrow flushed from the long bones of C57Bl/6 mice was treated with ammonium chloride to lyse red cells. Lymphocytes, granulocytes and Ia^+ cells were depleted by incubation with monoclonal antibodies (MAbs) 2.43 (anti-CD8; ATCC, Rockville, MD, USA), GK1.5 (anti-CD4; ATCC), RA3-3A1/6.1 (anti-B220/CD4SR; ATCC), B21.2 (anti-Ia; ATCC), RB6-85C (anti-Gr-1; Pharmingen, San Diego, CA, USA) and rabbit complement. The cells were plated in RPMI 1640 medium containing 5% heat-inactivated fetal calf serum, 10 mM HEPES (pH 7.4), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 500 U/ml recombinant murine GM-CSF (Boehringer Mannheim, Indianapolis, IN, USA). After 7 days, the tightly adherent monocytes were harvested for transduction, while the nonadherent and loosely adherent cells were collected and replated in 100-mm Petri dishes (8×10^6 cells per dish). The nonadherent cells were removed after 30 min by washing, and medium containing GM-CSF was added to the dish. The cells were incubated for 18–24 h and the floating DC population was then harvested for analysis and transduction.

Recombinant adenoviral infection

Ad.βgal and Ad.MUC1 are structurally similar replication-deficient recombinant adenoviruses in which the *LacZ* and *DF3/MUC1* genes,[22] respectively, are under control of the cytomegalovirus (CMV) immediate-early promoter and enhancer.[23,24] DC and monocytes were incubated with recombinant adenovirus at the indicated MOI for 6 h, washed and then cultured in medium containing GM-CSF.

Analysis of adenovirus-transduced DC

Cells were washed with PBS and incubated with MAb D19–2F3–2 (anti-βgal; Boehringer Mannheim), DF3 (anti-MUC1), M1/42/3.9.8 (anti-MHC class I; ATCC), M5/114 (anti-MHC class II; ATCC), 16–10A1 (anti-B7–1; provided by Dr Hans Reiser, Dana-Farber), GL1 (anti-B7–2; Pharmingen) or 3E2 (anti-ICAM; Pharmingen) for 30 min on ice. After washing, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-hamster, -rat or -mouse IgG for analysis by FACScan (Becton Dickinson, Bedford, MA, USA). For immunoperoxidase staining, the cells were centrifuged on to slides, incubated with MAb DF3 or MAb M5/114 and stained by the avidin–biotin complex method (Vector Laboratories, Burlingame, CA, USA).[25]

Mixed lymphocyte reactions

Control and transduced DC were treated with 20 Gy ionizing radiation. The cells were incubated at varying ratios with syngeneic (C57Bl/6) or allogeneic (Balb/c) T cells in 96-well flat-bottomed plates for 4–5 days. The T cells were prepared by passing spleen suspensions through nylon wool columns, incubating for 90 min in culture dishes and collecting the nonadherent cells. Stimulation of T cells was assessed by pulsing with 1 μCi/well ^{3}H-thymidine (New England Nuclear, Boston, MA, USA) for 6 h and monitoring for tritium incorporation.

Immunoblot analysis

Lysates from control and Ad.MUC1-transduced DC were subjected to electrophoresis in 6% polyacrylamide gels and analysis for reactivity with MAbs DF3 and DF3-P as described.[19]

Immunizations

C57Bl/6 mice were injected intravenously with 5×10^5 DC or monocytes on day 0 and again on day 10.

CTL assays

CTL activity was determined by the lactate dehydrogenase (LDH) release assay (CytoTox; Promega, Madison, WI, USA).[26] Splenocytes isolated from mice were subjected to Ficoll density gradient centrifugation. The splenocytes were incubated with target cells at varying E:T ratios in V bottom microtitration plates (Nunc, Roskilde, Denmark), centrifuged for 3 min at 1000 *g* and incubated for 4 h at 37°C. At the end of coculturing, 50 μl supernatant were transferred to an assay plate and incubated with 50 μl of substrate mixture for 30 min at room temperature. Absorbance was determined at 490–429 nm by microplate reader (model 3550, BIO-Rad Laboratories, Hercules, CA, USA). Killing of target cells by effectors was determined by the formula: cytotoxicity (%) = 100 × (experiment release − spontaneous release)/(maximum release − spontaneous release).

Antitumor activity

Mice were immunized twice (day 0 and 10) by intravenous injection of 5×10^5 DC or monocytes. On day 18, mice were challenged subcutaneously with 2×10^5 MC-38 cells that stably express DF3/MUC1.[22] Tumors >3 mm or greater in diameter as determined by vernier callipers were scored as positive.

Acknowledgements

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Breast Cancer Selective Gene Expression and Therapy Mediated by Recombinant Adenoviruses Containing the DF3/MUC1 Promoter

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Abstract

The high molecular weight mucin-like glycoprotein, DF3 (MUC1), is overexpressed in the majority of human breast cancers. Here we demonstrate that replication defective recombinant adenoviral vectors, containing the DF3 promoter (bp −725 to +31), can be used to express β-galactosidase (Ad.DF3-βgal) and the herpes simplex virus thymidine kinase (HSV-tk) gene (Ad.DF3-tk) in DF3 positive breast carcinoma cell lines. In vivo experiments using breast tumor implants in nude mice injected with Ad.DF3-βgal demonstrated that expression of the β-galactosidase gene is limited to DF3-positive breast cancer xenografts. Moreover, in an intraperitoneal breast cancer metastases model, we show that i.p. injection of Ad.DF3-tk followed by GCV treatment results in inhibition of tumor growth. These results demonstrate that utilization of the DF3 promoter in an adenoviral vector can confer selective expression of heterologous genes in breast cancer cells in vitro and in vivo. (*J. Clin. Invest.* 1995. 96:2775–2782.) Key words: adenovirus • breast cancer • thymidine kinase • β-galactosidase • gene therapy

Introduction

Gene therapy is a potentially novel approach to cancer treatment. In this context, transfer of suitable genetic material into a specific cell type (either tumor or host) can be used to alter the phenotype of the target cell. One such strategy is based on direct transfer of a "suicide gene" which encodes an enzyme such as herpes simplex thymidine kinase (HSV-tk)[1] that can activate a prodrug within tumor cells and thereby render the tumor cells sensitive to agents which are otherwise nontoxic to the cell. For example, ganciclovir (GCV) is a nucleoside that is nontoxic to mammalian cells, but is lethal after phosphorylation in cells that express HSV-tk (1–4). While gene therapy may provide a new therapeutic approach, clinical efficacy may require gene delivery systems which possess both high gene transduction efficiency and target cell specificity.

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1. *Abbreviations used in this paper:* Ad, adenovirus; CMV, cytomegalovirus; GCV, ganciclovir; HSV-tk, herpes simplex thymidine kinase; pfu, plaque-forming unit(s); X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside.

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Human adenoviruses are non-enveloped double-stranded DNA viruses with a genomic size of ~ 36 kb (5, 6). The E1 gene-deleted adenoviruses are replication defective and can be grown in a packaging cell line transformed with the E1a and E1b genes (7). Adenoviral vectors deleted at the E1 and E3 regions are capable of accommodating DNA inserts up to 8 kb (8, 9). Moreover, adenovirus-mediated gene transfer is a highly efficient means for delivery of genetic material into a wide spectrum of cells both in vitro and in animals. Although recombinant adenoviruses hold promise for in vivo gene therapy and are being tested clinically (10), one of the limitations of this vector system for cancer therapy may be the nonspecific transduction of therapeutic genes into nontarget cells. One strategy to circumvent this limitation would be to use a tumor-tissue specific/selective promoter or enhancer to direct the expression of a therapeutic gene in the desired target cells.

DF3 antigen (also designated MUC1 and episialin) is a member of a family of high molecular weight glycoproteins which are aberrantly overexpressed in most human breast cancers. We have previously shown that monoclonal antibody mAb DF3, prepared against a membrane-enriched extract of a human breast carcinoma metastatic to liver, reacts with over 75% of primary human breast carcinomas (11). Other studies have shown that overexpression of the DF3 gene in human MCF-7 (12) and ZR-75 breast cancer cells (13) is regulated at the transcriptional level. Recent cloning and characterization of the 5′ flanking region of DF3 gene has demonstrated that the DF3 gene expression is mainly regulated by sequences between positions −598 and −485 bp upstream to the transcription start site (14).

In the present work, we describe the construction of replication defective adenoviral vectors containing the *E. coli* β-galactosidase gene (Ad.DF3-βgal) or the HSV-tk gene (Ad.DF3-tk) under control of the DF3 promoter. We have evaluated expression of the reporter gene in DF3-positive and -negative cells in vitro and in vivo. We have also assessed the ability of Ad.DF3-tk to confer sensitivity to GCV in human breast cancer models in athymic nude mice. Our results suggest that an adenoviral vector system containing the DF3 promoter is capable of directing efficient and selective expression of heterologous genes in DF3-positive breast carcinomas.

Methods

Cell culture. The MCF-7, ZR-75-1, BT-20, and MDA-MB231 breast cancer cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD). The Hs578Bst myoepithelial cell line derived from normal breast tissue adjacent to a infiltrating ductal carcinoma (15) and the human T98G glioblastoma cell line were also obtained from ATCC. Cells were grown as monolayers in recommended culture medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained in a 5% CO_2 humidified atmosphere.

Recombinant adenoviruses. Recombinant adenoviruses Ad.DF3-βgal and Ad.CMV-βgal derived from type 5 adenovirus (Ad 5), were produced by homologous recombination in the human embryonic kidney cell

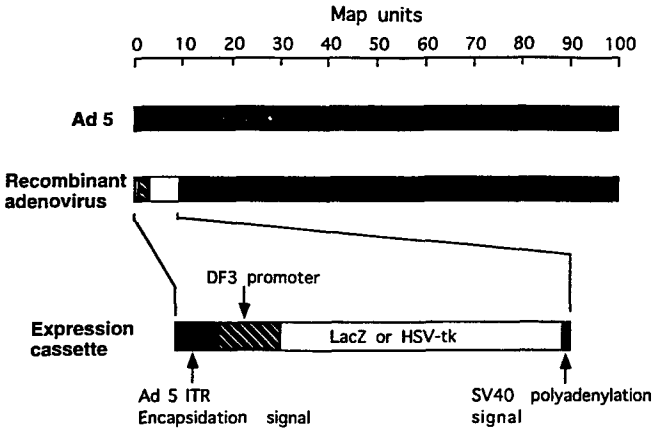

Figure 1. Schematic representation of the recombinant Ad.DF3-βgal and Ad.DF3-tk adenoviral vectors. Ad.DF3-βgal was constructed by insertion of a β-galactosidase (lacZ) expression cassette containing the DF3 promoter and SV40 polyadenylation signal at the deleted E1 region Ad 5 (1.3–9.2 mu). Ad.DF3-tk is a structurally similar adenovirus in which the lacZ gene is replaced by the HSV-tk gene.

line 293 (7). The DF3 5′ flanking region (−725 to −31) was inserted into XhoI and SpeI digested plasmid pCMVβgal (provided by Dr. R. Crystal, Cornell Medical Center, NY). The resulting plasmid pDF3βgal contains the E. coli β-galactosidase (lacZ) gene with the SV-40 polyadenylation signal under the control of DF3 promoter and SV-40 splice donor/acceptor signal, flanked by Ad 5 map units 0.0–1.3 and 9.3–17.3 (Fig. 1). To construct Ad.DF3-tk, a 2.0-kb cDNA of HSV-tk (16) was used to replace the lacZ gene in the shuttle plasmid pDF3βgal (Fig. 1). The shuttle plasmids were cotransfected by calcium phosphate precipitation into 293 cells together with pJM17 plasmid containing the adenoviral genome (kindly provided by Dr. Graham, McMaster University, Ontario, Canada). Recombinant adenovirus was isolated from a single plaque and expanded in 293 cells. The viral DNA was purified and analyzed by restriction enzyme digestion and by polymerase chain reaction (PCR). A pair of primers, 5′-TCTAGACTAGTGGACCCTAGGGTTCATCGGAG-3′ and 5′-AACTCGAGGATTCAGGCAGGCGCTGGCT-3′ was used to amplify and verify the presence of the DF3 promoter (−725 to −31) in the viral genome. Ad.CMV-βgal and AD.CMV-tk are structurally similar replication-deficient recombinant adenovirus in which the lacZ and HSV-tk genes, respectively, are under the control of cytomegalovirus (CMV) immediate-early promoter and enhancer. Large scale production of recombinant adenovirus was accomplished by growth in 293 cells and purification by double cesium gradient ultracentrifugation as described (17). The titers of purified adenovirus were determined by a spectrophotometer and by plaque assays.

Adenovirus infection in vitro. 24 h after plating, cells were infected with adenovirus at a multiplicity of infection (MOI) of 10–50. 48 h later or at a specified time post infection, cells were evaluated for the expression of the reporter gene or evaluated for sensitivity to GCV.

Assays for β-galactosidase. Histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal). Sections of fresh frozen tissue (12 μm) or cells were fixed with 0.5% glutaraldehyde in phosphate-buffered saline (PBS) containing 1 mM $MgCl_2$ for 10 min, rinsed with PBS, and then incubated with X-Gal (1 mg/ml), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$ in PBS for 4 h.

Fluorescence-activated cell sorting (FACS) analysis. (*a*) Indirect immunofluorescent analysis of DF3 antigen. Cultured cells ($1-2 \times 10^6$) were washed extensively with 1% bovine serum albumin (BSA) in PBS and incubated with mAb DF3 (1 μg/ml) or isotype identical control antibody mouse IgG (F-8765; Sigma Chemical Co., St. Louis, MO) at 4°C for 1 h, and then washed with 1% BSA/PBS. Cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F2012; Sigma Chemical Co.) at 4°C for 1 h, washed and analyzed on a Becton Dickinson FACScan. Intensity of fluorescence was determined for 10,000 cells and compared with the fluorescence obtained using a nonreactive immunoglobulin of the same isotype. (b) FACSGAL assay (18). Briefly, 1×10^6 cells were suspended in 50 μl of serum-free culture medium and warmed to 37°C. An equal volume of 2 mM fluorescein di-β-D-galactopyranoside (FDG; Molecular Probes, Eugene, OR) was added to each aliquot of cells. The cells and FDG were mixed rapidly and incubated for 1 min at 37°C. Thereafter, cells were washed once with 4 ml ice-cold PBS and maintained in ice-cold PBS until analysis on a Becton Dickinson FACScan.

Assays for GCV sensitivity in vitro. Adenovirus (Ad)-infected and noninfected cells were plated at 4×10^4 cells/well in six-well plates. GCV was added to the culture medium at various final concentrations (0–250 μM). After 6–7 d of incubation, cells were washed with PBS and trypsinized. The number of viable cells were determined by trypan blue exclusion. Cell number was also assessed using a colorimetric cell proliferation (XTT) assay that measures the mitochondrial dehydrogenase activity of viable cells. Results are expressed as a growth ratio of the number of cells in plates containing drugs as a percentage of that in the corresponding drug-free controls.

In vivo gene transfer to human breast cancer xenografts. Female athymic nude mice (Swiss-nu/nu, Taconic, Germantown, NY), 20–25 grams, were used. For mice bearing MCF-7 or ZR-75-1 tumors, a single pellet of 17β-estradiol (1.7 mg/60-d release; Innovative Research, Toledo, OH) was implanted subcutaneously one day before tumor inoculation. (*a*) Subcutaneous tumor model. Cells (MCF-7, ZR-75-1 and MDA-MB231) in exponential growth phase (1×10^7 in 0.2 ml) were injected subcutaneously in the flanks of the animals. At 4 to 6 wk after tumor implantation, up to 5×10^8 plaque-forming units (pfu) of purified recombinant adenovirus in 20 μl were injected into MCF-7, ZR-75-1, and MDA-MB231 xenografts, and into limb skeletal muscle. A Hamilton syringe with a 26-gauge needle was used for injection. The needle was coated with fine charcoal particles to mark the needle tract in order to verify colocalization of the expression of the reporter gene with the viral injection. 3 d after adenoviral infection, the animals were killed and expression of the reporter gene in tumor xenografts and host tissues was evaluated. (*b*) Intraperitoneal tumor model. MCF-7 cells (5×10^6) were injected i.p. for the development of intraperitoneal tumors (day 0). On days 4 and 5 after tumor cell injection, 1×10^9 pfu adenovirus in 0.5 ml PBS were injected i.p. into the mice. On day 7, the animals were treated with either saline or GCV (125 mg/kg) daily for 4 d by i.p. injection. The animals were killed at 5–6 wk after tumor inoculation. Tumors were collected and weighed for each animal.

Results

Selective expression of β-galactosidase in human cell lines in vitro. Expression of β-galactosidase was evaluated in Ad.DF3-βgal-infected MCF-7, ZR-75-1, BT-20, and MDA-MB231 breast cancer cells. In addition, Hs578Bst, a myoepithelial cell line derived from normal breast tissue and T98G, a human glioblastoma cell line were used in these studies. Each cell line was infected with either Ad.DF3-βgal or Ad.CMV-βgal at a MOI of 50. Expression of β-galactosidase was observed in MCF-7, ZR-75-1, and BT-20 cells infected with Ad.DF3-βgal (Fig. 2, *A–C*), whereas little if any β-galactosidase activity could be detected in similarly infected Hs578Bst, MDA-MB231 and T98G cells (Fig. 2, *D–F*). In contrast, all of these cell lines showed strong expression of β-galactosidase when infected with Ad.CMV-βgal (Fig. 2, *G–I*) in which the reporter gene is under control of the CMV early promoter and enhancer. MCF-7 cells infected with Ad.DF3-βgal at an MOI of 50 exhibited highest β-galactosidase activity at day 3 to day 7 post infection. Transgene expression gradually decreased to ~ 15% of maximum at 2 wk after infection (data not shown).

Expression of β-galactosidase in Ad.DF3-βgal–infected cells correlates with the expression of DF3. To assess whether

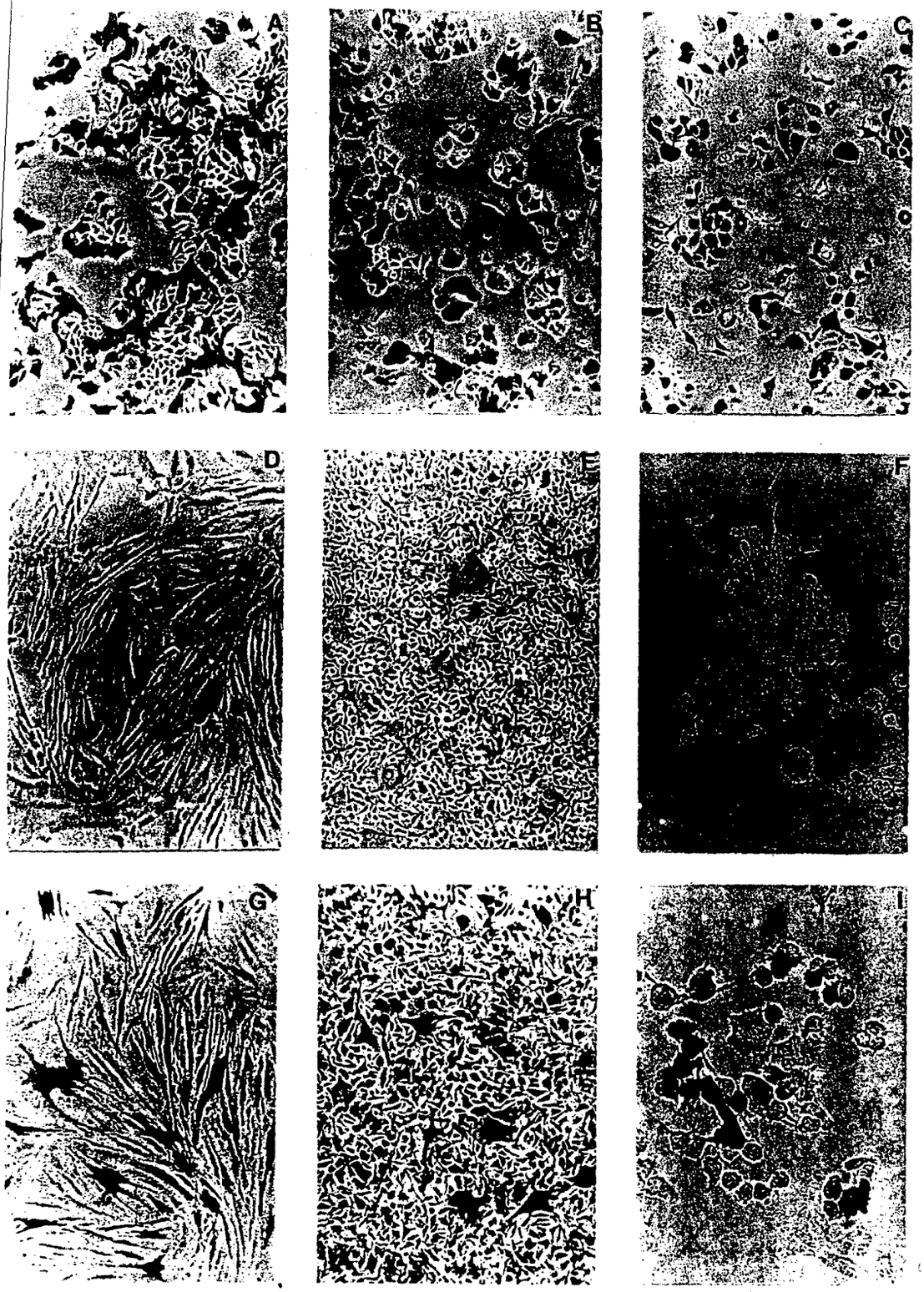

Figure 2. Expression of β-galactosidase in human cell lines infected with recombinant adenoviruses. Cells in exponential growth phase were infected with adenoviruses at an MOI = 50. 2 d after adenovirus infection, cells were fixed in 0.5% glutaraldehyde and stained with X-Gal for 4 h. MCF-7 (*A*, DF3+), ZR-75-1 (*B*, DF3+), BT-20 (*C*, DF3+), Hs578Bst (*D*, DF3−), MDA-MB231 (*E*, DF3−), and T98G (*F*, DF3−) cells infected with Ad.DF3-βgal. Hs578Bst (*G*), MDA-MB231 (*H*), and T98G (*I*) cells infected with Ad.CMV-βgal. All panels 100× magnification.

there is a correlation between DF3 expression and capability of these cells to express β-galactosidase after Ad.DF3-βgal infection, we examined the presence of DF3 antigen in MCF-7, ZR-75-1, and Hs578Bst cells by FACS analysis. MCF-7 and ZR-75-1 cells exhibited strong reactivity with mAb DF3, a monoclonal antibody against DF3 antigen, while little if any mAb DF3 binding was detectable with Hs578Bst cells (Fig. 3 *A*). Using the FACS-GAL assay, all MCF-7 and ZR-75-1 cells infected

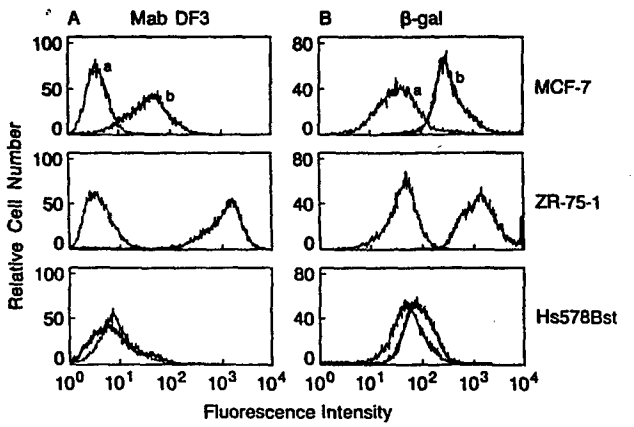

Figure 3. β-galactosidase expression in Ad.DF3-βgal infected cells correlates with expression of DF3 antigen. (*A*) FACS analysis to detect the presence of DF3 antigen in MCF-7, ZR-75-1, and Hs578Bst cell lines. Cells were incubated with mouse IgG as control (curve *a*) or MAb DF3 (curve *b*) and then FITC-conjugated anti-mouse IgG. (*B*) FACS-GAL assay to analyze β-galactosidase activity in uninfected cells (curve *a*) or Ad.DF3-βgal (MOI = 50) infected cells (curve *b*). *y*-axis indicates relative number of cells, *x*-axis shows fluorescence intensity.

with Ad.DF3-βgal appear to express β-galactosidase with an increase in mean fluorescent intensity of up to 35-fold (Fig. 3 *B*). In contrast, there was little if any expression of β-galactosidase in Ad.DF3-βgal-infected Hs578Bst cells (Fig. 3 *B*). These findings support a correlation between the presence of cellular DF3 antigen and expression of β-galactosidase in Ad.DF3-βgal-infected cells.

Ad.DF3-tk sensitizes DF3-positive MCF-7 and ZR-75-1 breast cancer cells to GCV in vitro. Given the finding that the DF3 promoter can direct selective expression of a reporter gene, we replaced the β-galactosidase gene in Ad.DF3-βgal with HSV-tk. To determine whether Ad.DF3-tk can confer sensitivity to GCV, MCF-7 and ZR-75-1 cells were transduced with Ad.DF3-tk at MOIs of 10 and 50. Ad.CMV-tk was used in order to assess HSV-tk gene expression under control of the different promoters. Infection with Ad.DF3-tk had little effect on cell viability (Fig. 4). Moreover, Ad.DF3-tk transduction conferred sensitivity of both MCF-7 and ZR-75 cells to GCV (Fig. 4, *A* and *B*), while nontransduced cells or cells transduced with Ad.CMV-βgal or Ad.DF3-βgal (data not shown) were insensitive to GCV (Fig. 4). As previously demonstrated (19, 20), similar results were obtained when cells were exposed to GCV for 6–7 d or to GCV for 24 h followed by incubation for 5–6 d in media (data not shown). The degree of Ad.DF3-tk–mediated GCV sensitivity was comparable to that obtained with Ad.DF3-tk. In contrast, when Ad.DF3-tk and Ad.CMV-tk were used to in infect DF3-negative Hs578Bst epithelial cells, only Ad.CMV-tk infected cells were sensitive to GCV (Fig. 4 *C*).

In vivo targeted gene expression in human breast cancer xenografts. To ascertain whether Ad.DF3-βgal can confer selective expression of β-galactosidase in vivo, we injected up to 5 × 10^8 pfu of Ad.DF3-βgal into MCF-7 and ZR-75-1 cells grown as subcutaneous xenografts in athymic nude mice. 3 d after adenovirus infection, the tumors were excised and assayed for β-galactosidase activity. Expression of β-galactosidase was distributed extensively along the needle tract of Ad.DF3-βgal injection (as indicated by the co-localized charcoal particles) in both MCF-7 and ZR-75-1 tumor nodules (Fig. 5, *A* and *B*). Blue staining was detectable within the tumor mass, but not in

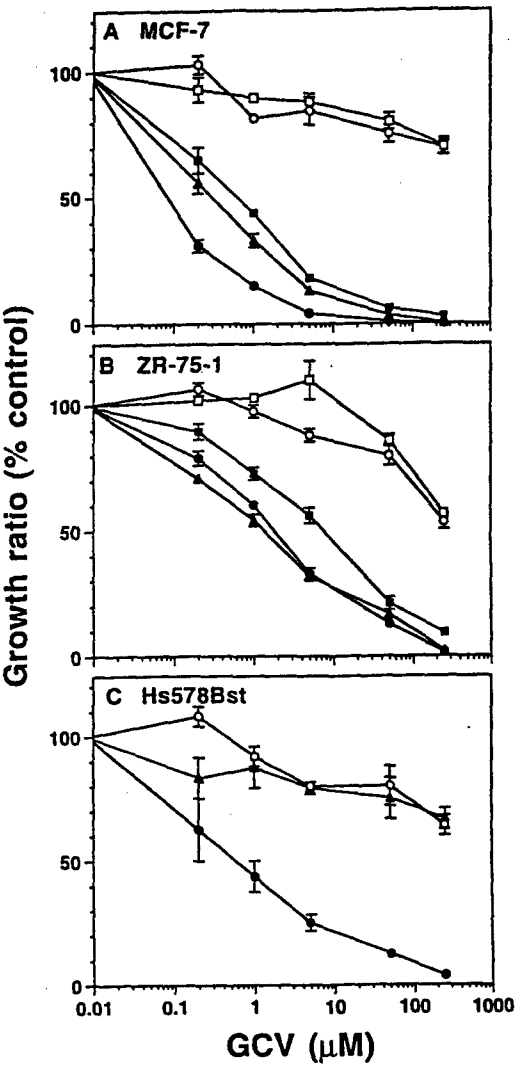

Figure 4. Ad.DF3-tk selectively sensitizes DF3-positive breast cancer cells to GCV. Cells were infected with Ad.DF3-tk or Ad.CMV-tk at MOIs of 10 to 50. Uninfected cells or cells infected with Ad.CMV-βgal were used as controls. 48 h later, cells were replated at 4 × 10^4 cells/well in 6-well plates. GCV was then added at concentrations ranging from 0 to 250 μM. After 6–7 d of incubation, viable cells were counted using trypan blue exclusion. The effect of GCV on cell survival was expressed as the growth ratio, i.e., cell number in wells containing drug as a percentage of the corresponding drug free control (mean±SE for duplicate determinations. (—□—) no adenovirus; (—○—) Ad.CMV-βgal (MOI = 50); (—■—) Ad.DF3-tk (MOI = 10); (—▲—) Ad.DF3-tk (MOI = 50); (—●—) Ad.CMV-tk (MOI = 50). Similar results were obtained in three separate experiments.

the surrounding normal tissue (Fig. 5 *B*). In contrast, when Ad.DF3-βgal was injected into DF3-negative MDA-MB231 tumors grown in nude mice, there was no detectable β-galactosidase staining along the needle tract (Fig. 5 *C*). Intramuscular injections of Ad.DF3-βgal also resulted in no detectable β-galactosidase activity (Fig. 5 *D*), while similar injections of Ad.CMV-βgal into skeletal muscle was associated with strong expression of β-galactosidase (Fig. 5 *E*).

β-galactosidase expression after systemic administration of Ad.DF3-βgal and Ad.CMV-βgal. To further evaluate adenovirus-mediated gene transfer to different tissues in vivo, 2 × $10^{\text{}}$ pfu of either Ad.CMV-βgal or Ad.DF3-βgal was injected via tail vein. Mice were killed 4 d after the injection and sections

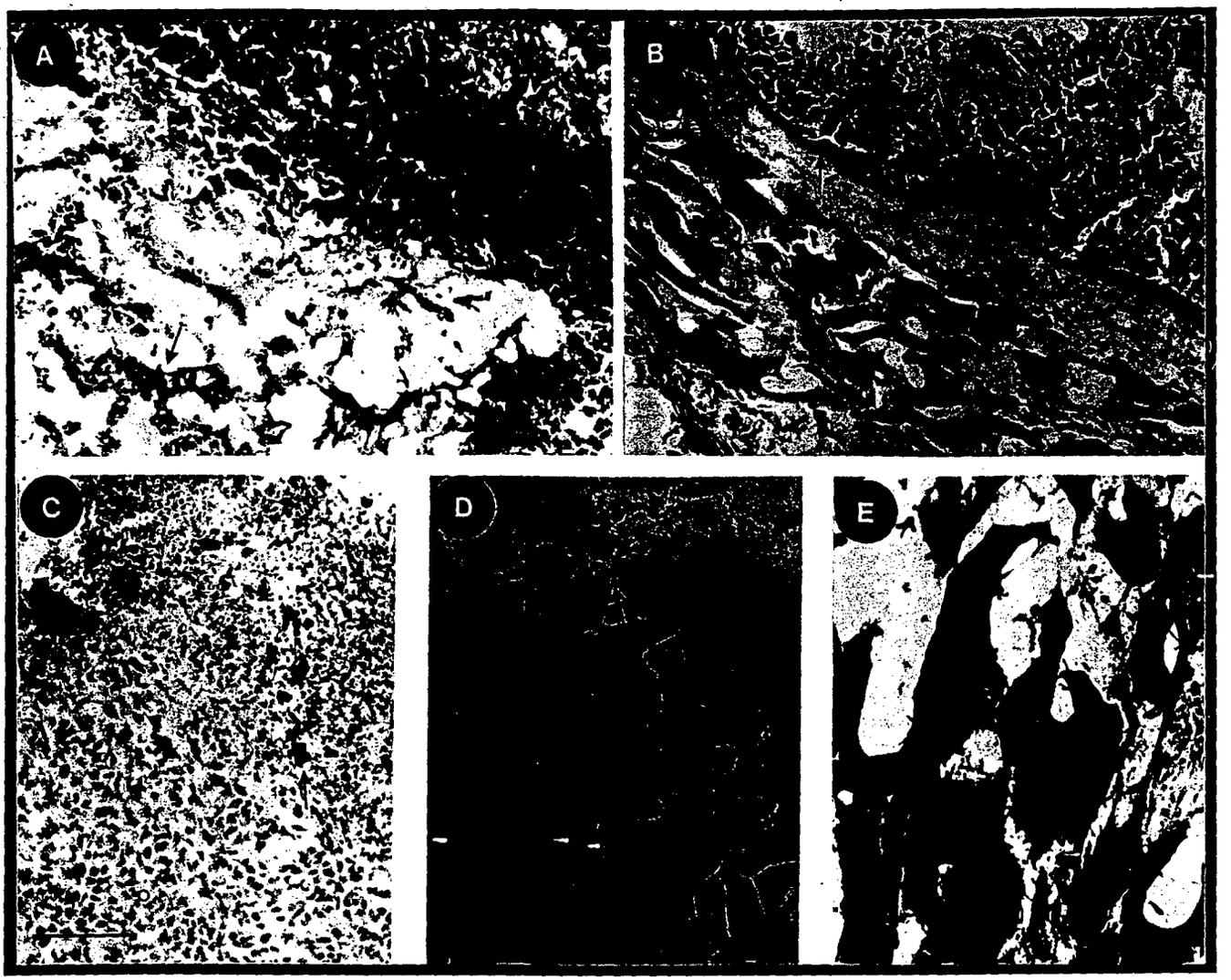

Figure 5. Ad.DF3-βgal mediated in vivo selective expression of β-galactosidase in breast cancer cells. MCF-7, ZR-75-1, and MDA-MB231 xenografts were grown subcutaneously in athymic nude mice. Adenovirus (5×10^8 pfu) was injected into the tumors or mouse limb skeletal muscle using a 26-gauge needle coated with charcoal particles (indicated by the arrowhead). The tissues were harvested 3 d after viral injection. The frozen sections (12 μm) were fixed in 0.5% glutaraldehyde and stained for β-galactosidase activity with X-Gal for 4 h. MCF-7 (*A*), ZR-75-1 (*B*), and MDA-MB231 (*C*) injected with Ad.DF3-βgal. Mouse skeletal muscle injected with Ad.DF3-βgal (*D*) or with Ad.CMV-βgal (*E*). All panels $\times$100. Bar, 100 μm.

of the liver, spleen, and lung were stained for β-galactosidase activity. Systemic injection of Ad.CMV-βgal resulted in expression of β-galactosidase in the liver parenchyma, in the splenic red pulp, and diffusely in the lung (Fig. 6 *A*). By contrast, there was no detectable β-galactosidase staining in these tissues in mice injected with Ad.DF3-βgal. Several foci of β-galactosidase staining (representing $< 0.1\%$ of cells in tumor cross sections) were detected in MCF-7 tumors of animals that received systemic Ad.DF3-βgal (Fig. 6 *B*). However, i.p. injection of Ad.DF3-βgal into mice bearing intraperitoneal MCF-7 tumor was associated with extensive expression of β-galactosidase in the tumor (Fig. 6 *C*).

Treatment of intraperitoneal breast tumor. To evaluate the therapeutic efficacy of Ad.DF3-tk in human DF3-positive breast cancer cells in vivo, nude mice were inoculated i.p. with MCF-7 cells. These mice developed tumor masses throughout the peritoneal cavity and 2–4 ml of bloody ascites that contained tumor cells (Fig. 7 *A*). MCF-7 tumor bearing mice were treated with Ad.DF3-βgal + saline, Ad.DF3-βgal + GCV, Ad.DF3-tk + saline and Ad.DF3-tk + GCV. Adenoviruses were injected i.p. on day 4 after tumor inoculation. GCV or saline injections were then administered on day 7 after tumor inoculation. Untreated mice and those treated with Ad.DF3-βgal or Ad.DF3-tk + saline developed multiple intraperitoneal tumors with bloody ascites. In contrast, there was no grossly identifiable tumor mass or only a few small tumor clusters with no apparent ascites in the Ad.DF3-tk/GCV-treated mice (Fig. 7, *B* and *C*). In other studies, Ad.DF3-tk (2×10^9 pfu) was administered intravenously by tail vein to mice with intraperitoneal tumors. The finding that treatment of these animals with GCV had no detectable therapeutic effect is in concert with the limited transduction of tumor cells after systemic administration of the vector (as shown in Fig. 6 *B*).

Discussion

The results presented here demonstrate that the DF3 gene promoter in the context of a recombinant adenoviral vector can

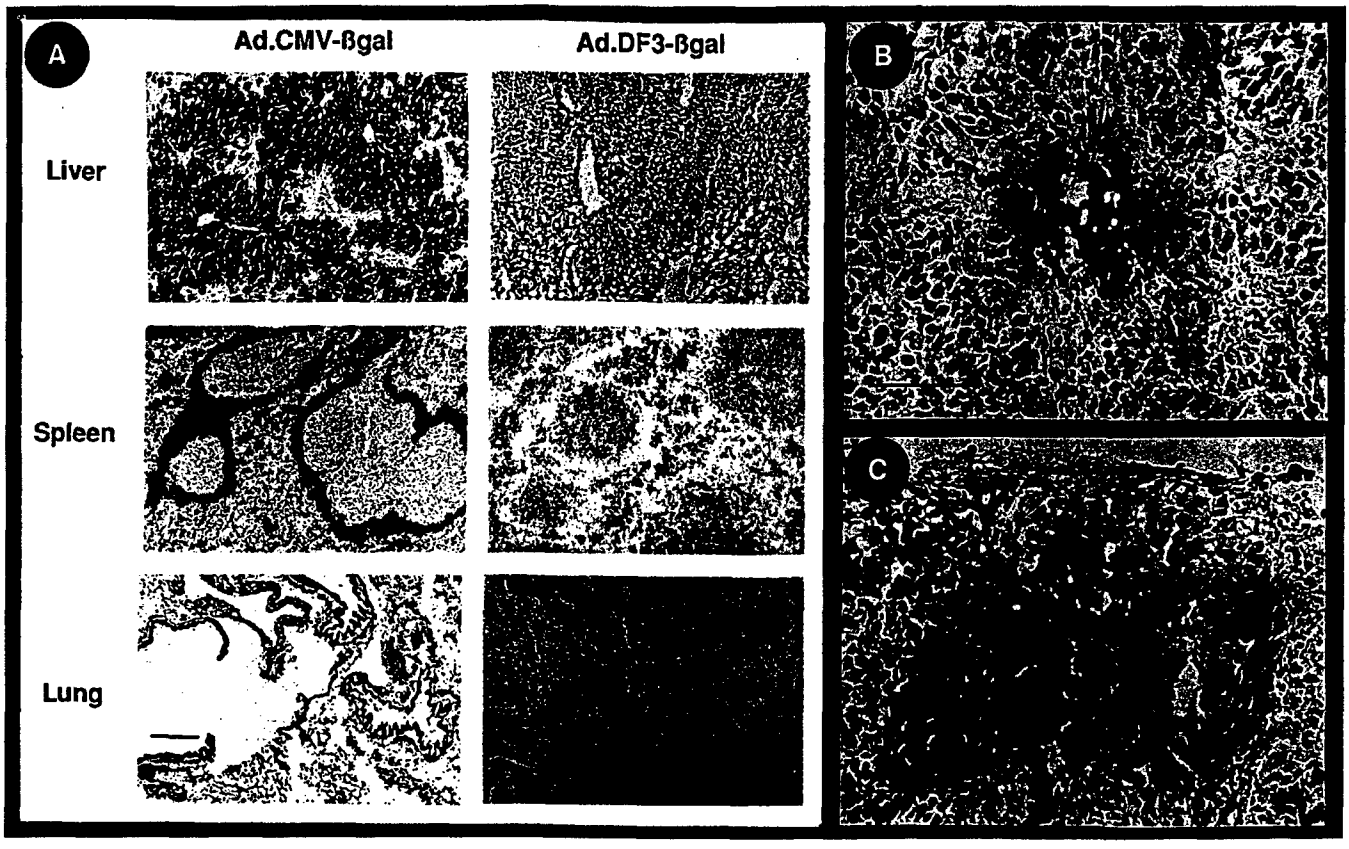

Figure 6. Distribution of β-galactosidase expression in mice injected systemically with recombinant adenoviruses. 4 d after adenovirus injection, sections (12 μM) of the individual tissues were prepared and stained with X-Gal for β-galactosidase activity. (*A*) Mice were injected with 2×10^9 pfu or Ad.DF3-βgal or Ad.CMV-βgal in 0.1 ml PBS through tail vein. Expression of β-galactosidase was evaluated in liver, spleen, and lung. ×40. Bar, 200 μm. (*B*) Mice bearing intraperitoneal MCF-7 tumors were given 2×10^9 pfu Ad.DF3-βgal intravenously. Tumor was evaluated for β-galactosidase expression. ×100. (*C*) Mice bearing intraperitoneal MCF-7 tumors were given 2×10^9 pfu Ad.DF3-βgal i.p. Tumor was evaluated for β-galactosidase expression. ×100. Bar, 100 μm.

confer selective expression of a reporter gene in DF3-positive human breast cancer cells in vitro and in vivo. Moreover, we demonstrate that adenovirus-mediated transduction of HSV-tk under control of the DF3 promoter can confer selective sensitivity of DF3 positive cells to GCV. These findings support the potential use of tumor-selective promoters to target expression of therapeutic genes in adenovirus-mediated gene therapy.

Currently available in vivo gene delivery systems generally lack target cell specificity. Nonviral approaches, such as ligand-DNA complexes mediated gene transduction through receptor endocytosis have been reported to deliver genes through asialoglycoprotein receptors (21, 22). This approach, however, is largely hindered by the relatively low gene transduction efficiency due to the endosomal/lysosomal trapping of the DNA complex. Although other approaches such as the use of DNA liposome complexes (23, 24) and direct injection of DNA (25) have been applied for direct in vivo gene transfer, they are also limited by relative low gene transduction or restricted target tissues. Retroviral vectors have been used extensively for gene transfer, particularly for ex vivo gene therapy (26–29). However, application of retroviruses for in vivo gene therapy may be limited by low viral titers and the dependence on target cell replication. In addition, random chromosomal integration of retroviruses may raise some potential safety concerns.

Recently, recombinant adenoviruses have been employed as a highly efficient approach for in vitro and in vivo gene transfer. Adenovirus-mediated gene transduction is independent of cell replication and the virus is capable of infecting a broad spectrum of eukaryotic cells. However, one limitation of this vector system may be nonspecific transduction of therapeutic genes into cells other than the target cells. This limitation may be critical when "suicide" genes are being delivered into tumors. Therefore, one potential strategy is to design an adenoviral vector in which a therapeutic gene can be expressed selectively in tumor cells with little or no expression in normal cells. Although adenovirus infects a wide range of cells, the present studies demonstrate that expression of a heterologous gene under the control of DF3 promoter in the context of an adenovirus vector is limited to DF3-positive breast cancer cells. Another limitation of adenoviral vectors is the induction of an immune response that precludes prolonged transgene expression and repeated administration. The development of an immune response to adenovirus, although probably limited in athymic mice (30), could also contribute to the antitumor activity associated with tk transduction and GCV therapy. Thus, studies in an animal model such as the one used in the present work may provide only certain insights into the eventual use of adenoviral vectors in humans. In addition to the E1-deleted adenoviruses, the development of new generation vectors by deleting E2a (31) or E4 (32) may further limit induction of an immune response. Finally, systemic administration of adenoviral vectors is associated with predominant transduction of hepatocytes (33–35).

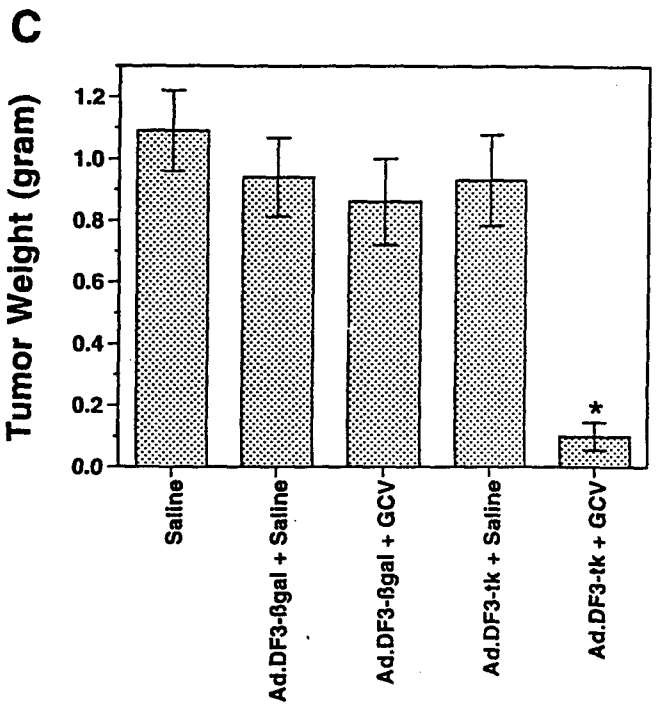

Figure 7. Treatment of intraperitoneal MCF-7 breast tumors. MCF-7 cells (5×10^6) were inoculated i.p. into nude mice. On days 4 and 5, mice received i.p. injections of Ad.DF3-tk or Ad.DF3-βgal (1×10^9 pfu) in 0.5 ml PBS. GCV treatment was initiated on day 7 at a dose of 125 mg/kg body weight daily via i.p. injection for 4 d. 6 wk after GCV treatment, animals were killed for evaluation. (*A*) Intraperitoneal tumor in an untreated mouse, showing multiple solid tumors (*arrows*) and bloody ascites. (*B*) Mouse treated i.p. with Ad.DF3-tk/GCV showing no evident intraperitoneal tumor. (*C*) Tumor masses from each group were collected and weighed (mean±SE, $n = 6$). In the Ad.DF3-tk + GCV treatment group, two mice had no grossly visible tumor. Similar results were obtained in three separate experiments. Asterisk, significance ($P < 0.001$) compared with other groups.

The similar findings obtained in our studies further support the present limitations in using adenoviral vectors for the treatment of a disseminated tumor. These findings also support the potential need for promoters that are not activated in hepatocytes.

To date, only a few tumor tissue-specific or -selective DNA regulatory sequences have been identified. Moreover, most studies of selective promoters to target tumor cells were performed in cell culture or ex vivo models. Selective expression in melanoma cells has been reported using plasmids containing the promoters of the genes encoding tyrosinase and tyrosinase related proteins (36, 37). The promoter of the α-fetoprotein gene (38), the promoter of the oncogene ERBB2 (39), and recently the enhancer sequence (−598 to −485) of the DF3 gene (20) have been explored in the context of retroviral vectors to direct expression of prodrug activating enzymes and to confer selective tumor killing in a number of cancer cell lines in vitro. However, to our knowledge, there are no previous reports of using a tumor-selective promoter in the context of an adenovirus vector to selectively kill tumor cells in vivo. In the present studies, we have demonstrated an efficient and selective approach to target expression of heterogous genes in DF3-positive breast cancer cells. Currently, recombinant adenoviral vectors containing the DF3 promoter and other candidate therapeutic genes including cytochrome P450 (40, 41), and cytosine deaminase (42, 43) are being developed.

Acknowledgments

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